ANNUAL REVIEW OF BIOCHEMISTRY

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PREFACE

The Editorial Committee of this *Review* meets annually to select the topics and the authors for the volume to be published two years later. In the good old days of 20 years ago the selection of topics was not difficult. It was an accepted fact that the subject matter of biochemistry could be divided into discrete clusters of facts. Each cluster would grow larger from year to year, it would bulge and it would swell, but it need not necessarily divide into daughter clusters, nor need neighboring clusters fuse and rearrange. Proteins need not be confused with peptides; lipide metabolism need not spill over into carbohydrate metabolism; vitamins, enzymes, and hormones could be kept in separate packages; and so on. This has been a useful practical hypothesis which has long enjoyed a tongue-in-cheek acceptance. It has permitted departments of biochemistry in medical schools to adhere to substantially the same lecture and laboratory topics the world over; and it has permitted textbooks to grow larger and the chapter headings to remain the same.

Within this *Review* acceptance of the same philosophy was almost inescapable. Indeed, in the preface to Volume 3 (1954) we referred to topics "which by universal consent constitute the traditional divisions of the subject," topics which "will continue to receive review at annual or biennial intervals."

Our faithful adherence to so simple a policy has been shaken by the dramatic growth of the whole corpus of biochemistry and by the turmoil within. With the founding of other *Reviews* we have attempted to cut things back to size by the transfer to several sister volumes of topics that seemed more properly oriented toward physiology, microbiology, and plant physiology. But the truncated residue continues to grow and constantly taxes the best efforts of our authors to keep it within their compass. We devote more space to vitamins only to find that proteins, peptides, enzyme systems, protein biosynthesis, nucleic acids, and nucleotides are equally in need of more and more pages in the *Review*. Lipide metabolism, carbohydrate metabolism, and indeed, several groups of metabolic systems can no longer be kept discretely apart: one-carbon and two-carbon biochemistry has interpenetrated the entire metabolic framework.

It is this restless dynamic character of the subject that helps to bedevil the author, to tax his ingenuity, and to create demands upon him that can hardly be met. No longer is it easy to define with precision the boundaries of an assigned topic, to keep a review within the allocated space, or to encompass more than a fraction of the pertinent literature. Many papers are necessarily omitted because of the exigencies of space, because of the plausible assumption that another author may find them to be of greater relevance to his own topic, and because of the insistence of the editors that the reviews shall be critical appraisals and not synoptic summaries.

A good review, says Gutman[1] is a

> balanced and seasoned integration of significant developments in a chosen field, putting things in proper perspective, and finding a meaningful design where confusion reigned before. The construction of a good review requires an unusual background of experience and information, objectivity combined with imagination, clarity with vision—a combination of qualities not too widely possessed. Good reviews by talented reviewers are now more needed than ever; more assembly and less manufacture is required in many areas, so cluttered are our scientific journals with disjointed facts and figures.

Our gratitude to our reviewers cannot be adequately expressed; like the *Review* itself, it grows steadily. We would also thank our editorial assistants, especially Adele Fumino, who has carried the major responsibility of seeing this volume through from the beginning to the end. We acknowledge gratefully the generous assistance of Dr. Donald Kupke and Carol Kupke in preparing the subject index. To the rest of the staff and to the George Banta Company, Inc., we express our appreciation for their constant cooperation.

F.W.A. T.H.J.
F.S.D. J.M.L.
W.Z.H. G.M.
B.L.H. E.L.S.

[1] Gutman, A. B., "The Forms of Medical Writing," *Mississippi Valley Medical Journal*, **77**, 35 (1955).

Dr. Harry J. Deuel, Jr.

Dr. Harry J. Deuel, Jr., member (1946–56), President (1953–56), of the Board of Directors of Annual Reviews, died on April 17, 1956, a few hours after he had been elected President of the American Institute of Nutrition. The death of Dr. Deuel, an outstanding investigator, teacher, writer, and administrator, was a great loss to biochemical science and the academic profession.

Harry Deuel was a man of great sincerity, highly dedicated to the ideals of science. He possessed boundless energy and enthusiasm. He lived a very active life that was filled with remarkable achievements. Warm affection for, and deep loyalty to, his friends and fellow workers were evident in all of his actions. The many positions of high responsibility he held in academic institutions and professional organizations, his long list of publications, 283 in all, his many responsible tasks of public service, the countless personal services he so generously provided for his friends, all attest eloquently to his great devotion to his fellowman.

Deuel was born in St. Paul, Minnesota, on October 15, 1897, the son of Harry James and Myrtle Lillian Deuel. He received the A.B. degree in 1918 from Carleton College, and the Ph.D. degree in 1923 from Yale University. He served as Instructor (1923–27), and Assistant Professor (1927–28), of Physiology in Cornell University Medical College. During the academic year 1928–29 he was Professor of Physiology in the University of Maryland Medical School. In 1929 he went to the University of Southern California where he served as Professor of Biochemistry and Chairman of the Department until 1949, when he became Dean of the Graduate School. During 1955 and early 1956 he lectured in Europe under a Fulbright fellowship.

Deuel was a pre-eminent authority upon the lipides. His monumental treatise of three volumes on the chemistry, metabolism, and nutritive value of lipides will long serve as an authoritative text and reference. His work on the nutritional adequacy of fortified margarine, on isopropyl and stearyl citrates as preventatives of flavor reversion, on sorbic acid as a fungistatic agent in food packaging, and other similar studies decisively influenced legislation and practices in the food industry.

For his work in nutrition, Deuel was granted the Borden Award in 1949 by the American Institute of Nutrition. In 1954 he received the Monsanto Award given by the Institute of Food Technologists for excellence in presentation of a scientific paper. In August, 1955, he was presented an Honorary Award by the University of Brussels for achievements in the field of medicine.

JOSEPH H. ROE

ERRATA

Volume 25

page vii, line 3: *for* entrophy *read* entropy

page 513, line 10: *for* As much as 68 per cent of ζ-tocopherol and 16 per cent of ε-tocopherol *read* As much as 16 per cent of ζ-tocopherol and 68 per cent of ε-tocopherol

page 514, lines 39 and 40: *for* [Christensen & Dam (262a)] *read* [Christensen, Gortner & Dam (262a)]

page 531, reference 262a: *for* Christensen, R. A., Jr., and Dam, H. *read* Christensen, F., Gortner, R. A., and Dam, H.

page 541, line 18: *for* South America *read* Colombo, Ceylon

page 673, line 3: *for* now *read* not

Author Index

page 740: *for* Christensen, R. A., Jr., *read* Christensen, F.

page 746: *insert* Gortner, R. A., Jr., 514

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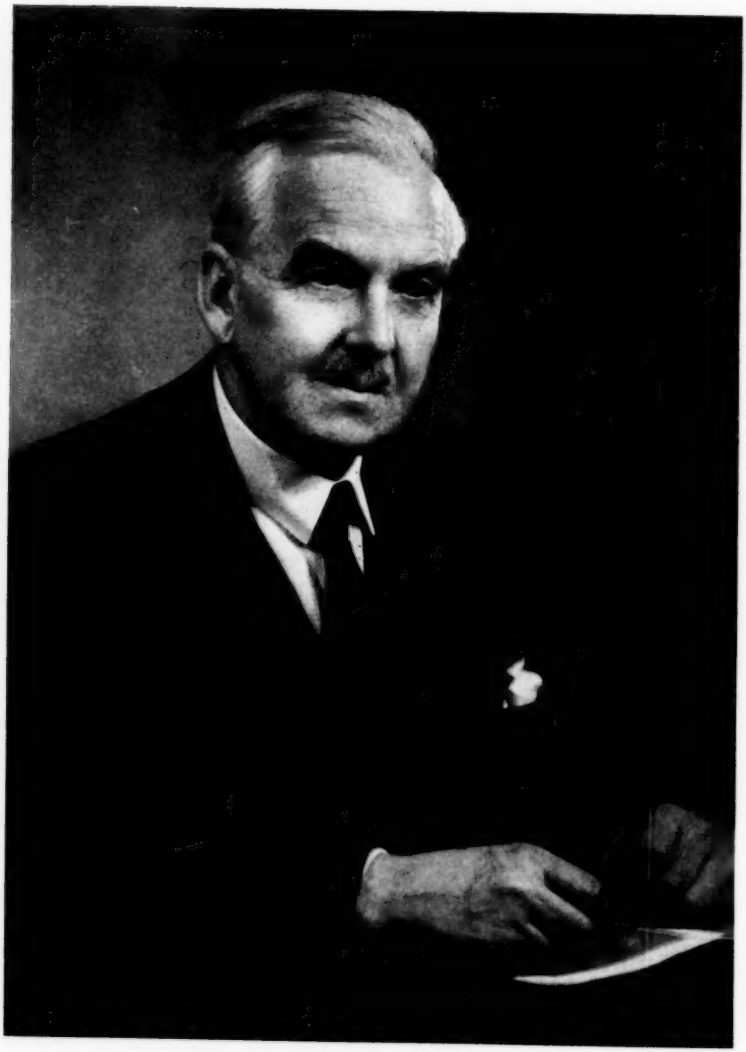
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Rudolph A. Peters.

PREFATORY CHAPTER
FORTY-FIVE YEARS OF BIOCHEMISTRY

By Sir Rudolph Peters[1]

Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Cambridge, England

It is never very easy for one brought up in an English public school especially, or even for one brought up in the British Isles, to be egotistical to the extent of describing how his way of life has come about. In part this is shyness, but in part also there is an element of doubt as to whether it can be of interest to anyone else. Still, as the Editors of this distinguished *Review* have thought it might be of value, I must try to do my best and hope that their confidence will not be misplaced.

Of an early life spent twixt school and two pleasant and small English country towns in which my father practised as a doctor, I started on my more serious career. At School the headmaster considered that, as I was headed for the profession of medicine like those two who have preceded me in writing these articles, I should be brought up on the classics, perhaps also because I had entered as a Classical Scholar. I realise that I owe an interest in literature and accuracy to my classical training, but it is curious that I was not allowed to do some science until the age of 17 or 18. Fortunately, perhaps, this drove me into science as a hobby, and I found myself reading avidly books on electricity and magnetism, and upon the more chemical sides of photography. During this time too I had the very good fortune to become friendly with one of the masters, S. A. Saunder, at Wellington, who was a distinguished astronomer; and many hours I spent in his company looking at the stars and discussing his work upon the moon; his task was to make as complete a map of this as possible. There is no doubt that in this way I was able to supplement the classics with a reasonable amount of science. At one time indeed I felt much attracted to astronomy!

Once away from school, however, life began to open out. The year of 1907 to 1908 found me a student for one year at King's College, London University, whither I had been sent to wait for an admission to Cambridge; no vacancy was available the year I left because it was a last-minute decision to send me to Cambridge. Still, it was at King's (in the Strand) that I started in earnest to prepare for my 1st M.B. at Cambridge. King's College had an excellent group of teachers: Jackson in Chemistry, Wilson in Physics, Dendy in Zoology, and Bottomley in Botany. All of them were stimulating, but especially can I remember the enthusiasm which inspired the lectures of Bottomley.

[1] Emeritus Professor of Biochemistry, Oxford University, and Honorary Fellow of Gonville and Caius College, Cambridge.

From King's College I went to Cambridge, to Gonville and Caius College. Here, immediately, scientific life began in earnest. My directors and teachers in physiology were the inspiring neurophysiologist H. K. Anderson[3] and W. B. Hardy, that remarkable man (a master mariner) who became Secretary of the Royal Society, and whose investigations ranged from early work on fixing gelatin films right through colloids and protein chemistry to finish up with surfaces and lubrications. He was, I believe, one of the few if not the only man to have given both the Bakerian Lectures and Croonian Lectures at the Royal Society, each being considered to be the outstanding lecture on the physical and biological sciences respectively. These two men could not have failed to interest us in physiology, and it was Anderson who introduced me to that monumental work by Sherrington, *Integrative Action of the Nervous System*. It is notable that the great physiologists Harvey and Sherrington should both have been undergraduates at Gonville and Caius College, and both also have spent some time in Oxford. I was much attracted also by the chemistry which I did at Cambridge, though this was not so tied to personalities as in the case of physiology. After Part I of the Natural Sciences Tripos which I took in 1910, two years after I had been at the University, it was decided that I should spend two years on the then Physiology Part II. During this period I was "roped" into his entourage by Joseph Barcroft (who was to become Sir Joseph and Professor of Physiology in succession to J. N. Langley). In that old physiological laboratory in which there was no room except the Professor's which was not a passage, Barcroft then worked behind a green baize curtain. I do not think that anyone quite knew how Barcroft started them off in research. Usually it commenced with his saying "Will you hold this?" or something of that kind. But I know that I found myself embarked on determining the relation between iron and oxygen in haemoglobin, using Barcroft's new differential gas analysis technique, which he taught me, for the determination of combined oxygen in blood. The iron estimation part of it I had to do much more alone, but I got introduced to the metallurgist, Heycock, probably somehow through Barcroft. Heycock was chiefly known to students in Cambridge through his lectures to Part I and his very vivid personality; some of his pithy criticisms were put in a way which students never forgot. He placed at my disposal the new method (very new in 1911) of estimating iron with titanium. I was also enabled to use his standard solutions and learnt from him that I must not heat the iron too much in the haemoglobin, or else it became insoluble. Out of this research came the chemical proof that the oxygen and iron were in stoichiometrical relation. The so-called specific oxygen capacity (O_2/Fe ratio) was always a little low, about 2 per cent, but I have been much encouraged by the fact that a high-powered group working upon the problem of haemoglobin estimation under the Chairmanship of Sir Charles Harington, as late as World War II, also found this same low value, using the titanium method. I think it is fair to say that my work settled the relation between iron and

[3] Subsequently Master of the College.

oxygen, and in proving the stoichiometric relation thereby provided a proof that, though the oxygen was dissociable, it was nonetheless a chemical relationship. Hence this was the first time in which it had been shown that this was not an adsorption, and that a colloid could take part in a strictly chemical reaction. During the course of these two years, I had the stimulus of various teachers, at what was the most heroic period of the old laboratory of physiology in Cambridge. It included in addition to Barcroft and Professor J. N. Langley (who was naturally somewhat dissociated from all of us), Keith Lucas, Walter M. Fletcher (afterwards first Secretary of the British Medical Research Council), and even the great Gaskell with his long beard (looking like Neptune when he lectured with his pointer held as a trident); this was the Gaskell who did so much to put the physiology of the heart upon a right basis. On and off I worked in that laboratory until 1914; Adrian[3] was in the passage which formed the small lecture preparation room, and was separated from myself by a small partition. There were in the laboratory then not only those I have mentioned, but several colleagues of Barcroft, among whom I have a vivid impression of Hamilton Hartridge, who was always a wizard with apparatus. In that laboratory too was Mines, a very brilliant young man whose early death deprived physiology of a most promising future, and A. V. Hill, of whom I will say a word later; and I remember that J. Crighton Bramwell, who has now become a famous heart specialist, was working with Keith Lucas. In some ways I may be said to have carried on in that laboratory from the time that E. Mellanby, whose article appears in last year's *Annual Review*, left it. There could have been no finer experience for any young man; the laboratory conditions by modern standards were dreadful, but it diverted several of us from medical practice into research.

After finishing this year with J. Barcroft, I thought it right to spend some research time in other ways. I had then a research studentship from my College. It must be remembered that in those days studentships were few and far between. There were no grants from the Government, the Medical Research Council, the Agricultural Research Council, or the Department of Scientific and Industrial Research. Nor were there Rockefeller fellowships and the like; we had to exist on smaller studentships picked up from our College (and mine was particularly generous). We also coached our way through and demonstrated in physiology for Langley. I believe that I even demonstrated anatomy! In 1912, therefore, I joined A. V. Hill to do a year's research with him in the heat and muscle field which he was then making his own. The work was upon the relation of heat production and lactic acid formation to stimulation of nerve in frog muscle. During this time he taught me, in addition to his approach, some thermodynamics, a slight amount of mathematics, and at any rate something about galvanometers, for which I have always been grateful. At the end I became elected to the new Benn Levy Fellowship in Biochemistry, of which I believe I was the first holder.

[3] Now Lord Adrian.

I went to work with Barcroft on blood problems again and was immersed in questions of estimating pH of the blood, being involved in modifications of the early technique of Hasselbach. We got out some relations between the main variables concerned, which were extended and considerably amplified later by the work of those at the Rockefeller Institute, viz. Van Slyke, Hastings, and their colleagues. Recently Professor Baird Hastings, when in Oxford as Fulbright Professor, recalculated some of the results which we obtained for pH in those primitive days, and I was relieved to know that our value for blood (I think it was Barcroft's) was only 0.15 pH more acid than it should have been! I cannot remember that we knew much then about lactic acid and glycolysis. The surprise is that it could ever have been as near as that; even the constants for the hydrogen electrode were not properly laid then. At that time, I know that I wasted a very great deal of research effort, trying to find an oxygen electrode. It was clearly one of those researches which was not going to come off, but it took me eight or nine months to realise this. I do not believe that there is even yet a satisfactory oxygen electrode. The ease with which it is possible now to do pH estimations with the glass electrode is still astonishing to me. Even in the early days of the glass electrode (in the 1930's as I remember), when Dr. Kerridge was working at this in London at University College, and Dr. R. E. Havard in my laboratory in Oxford, it used to take approximately a week to get the apparatus in order.

Following my Research Fellowship at Caius, in the early part of 1914, I even worked for a month in the new laboratory of physiology which had just been built for Langley by the Drapers Company (I believe with some assistance from the Beit Trustees). It was at that time in 1914 that Hopkins was given the old physiological laboratory to develop for Biochemistry. It is extraordinary that Cambridge never did anything positive for Hopkins, much as they loved and respected him, until he was on the eve of going somewhere else. I have always understood that he obtained his professorship finally at this time because an offer had been made for him to go elsewhere. It is quite true, as Edward Mellanby said in his article last year, that the first Chair of Biochemistry in this country was made in Liverpool for Benjamin Moore, once described by Hopkins as "that wayward genius." It is very interesting to note that not only the Chair of Biochemistry in Liverpool, but also the subsequent one in Oxford, were the results of the efforts of the physiologist, Sherrington, and that, both in Liverpool and in Oxford, E. Whitley had the vision to provide finance for biochemistry; in fact he also financed the *Biochemical Journal* in its early days.

After the month in the physiology laboratory, World War I descended upon us. This became an interlude for many; in my case it meant the finish of a medical education up in London at St. Bartholomew's Hospital, as well as taking rather an active part in matters connected with the Officers Training Corps. After qualifying in medicine in the summer of 1915, I was immediately sent down to R.A.M.C. work on Salisbury Plain, the training of

an ambulance unit; but it was not until the end of 1915 that I was sent out to France with this new Field Ambulance, with a Regular as C.O. We were about in France for six months, after which I was attached to the 1st Battalion of the 60th Rifles for nine months, as a battalion medical officer. Though naturally there were uncomfortable times and though it was most harrowing to see so many of the finest of our friends succumbing to the war, and though it was a serious interruption of a scientific life, I believe in retrospect it was a valuable human experience, and the medical responsibility, though narrow, gave some insight into the outlook of the clinician. After I had been in France for approximately 18 months, in the early part of 1917, Barcroft and others came to the conclusion that I might be of some use in helping them in research on problems of gas warfare, which had then become somewhat urgent. Accordingly, after a short passage through some of the units of the Chemical Corps, where I met C. G. Douglas at Headquarters in France, I was recalled to Porton, on Salisbury Plain, to the newly developing experimental station which has become now the Chemical Defence Experimental Establishment. There I spent the remainder of the war. Gas warfare is certainly a disagreeable and horrible affair, but it is quite illogical to consider it more disagreeable and more horrible than the shattering and maiming effects of high explosives. For those engaged in physiology and biochemistry it was an exciting though anxious time because the use of gas was then being developed by the enemy; in their turn we were introduced to mustard gas, phosgene, some of the arsenical compounds, and the arsenical smokes; and the solution of the problems of defence lay in our field. It is clear to me that the experience that I gained there, working with Barcroft, the pathologists Boycott and Shaw Dunn, and several chemists, orientated quite a good deal of my subsequent outlook. I realised clearly that there was not only an important piece of biochemistry and physiology to be done, but, at the same time, if the mechanism of action of these gases could be understood and counteracted, this might go a long way to preventing this misuse of the wonders of chemistry in the future.

In 1918, after the war, it was mooted that I should take a post at Porton; but I returned to my Fellowship at Cambridge. I came back, therefore, to the work at Caius, looking after the students, especially the medical students, who were rather numerous then. At the same time Langley suggested that I should look after the histology classes. It was not long afterwards that Hopkins told me that he was also looking for somebody, and as my real interests so far as I could understand them then lay in biochemistry, I naturally took this offer of Hopkins. The life in term was a busy one; not only was there teaching and administration in the Biochemistry Department until about 5 o'clock, but after that came the time in College looking after the students, and their teaching in the general subject of physiology. This, therefore, relegated research to the vacations and to those terms in which my teaching duties were reduced by Hopkins, or were not so heavy. At this time I got interested in two or three distinct subjects: the first was pharmacologi-

cal, a follow-on from the attempt to get some general rules as to how these gases worked, in other words to make it all less empirical. This led to an interest in the culture of the protozoa, a problem which lasted me some time, because I isolated a species of what was then known as *Colpidium* (now *Tetrahymena geleii*) which I kept going until somewhere in the 1940's. Certain things were right about these researches and some wrong, but they gave me a real insight into the complexities of the microorganism, because the protozoan is like a bacterium, only on a much enlarged scale. At the same time, the gas work again made me take an interest in surfaces, a subject in which W. B. Hardy was then actively engaged. I think he must be considered as the first to put forward ideas on the orientation of molecules in surfaces, and it was he who first introduced me to the papers of Langmuir. Even now I can remember the thrill with which I read those two classical papers. Following on these, with H. Hartridge, I embarked upon a research upon the effect of pH on interfaces between oil and water. This research again has lasted as an interest to which I have returned several times. Later on, in the 1930's, I published papers on these subjects. It was about 1922, arising from the attempt to grow *Paramoecium* with yeast extracts (and I expect largely through the influence of Hopkins), that I became interested in the isolation of the so-called Vitamin B, subsequently called Vitamin B_1, and now thiamine.

My duties in College left me comparatively little leisure, but I gained much from the experience of this Part II teaching and from the association with various folk in Hopkins' laboratory, both staff and visitors, of whom one of the latter is the Editor of this *Review*. So many of them were, or became, leaders in biochemistry. There was always the presiding influence of Hopkins which went very deep: I daresay deeper than some of us could have realised then. It was so well described by E. Mellanby last year that I do not think I need say much more; and it is of course very well put forth in the book entitled *Hopkins and Biochemistry*. For myself, these three interests, the one on surfaces, the other a pharmacological interest arising from the war gas field, and that in the nutritional field arising from thiamine, were threads which ran through my life for a very considerable time.

I do not think I have ever been more astonished than I was to receive a telephone call from Hopkins early in 1923 to say that he was empowered to offer me the Chair of Biochemistry at Oxford. Such a thing had never entered into my head. I was then only 33; I had not even known that he was going to an electoral meeting. As a result, April, 1923, saw me installed in Oxford as the new Professor of Biochemistry, a post which I was to hold for 31 years, with the donor of the Chair, Mr. E. Whitley, as one of my Demonstrators and with the friendly Trinity College as my new Academic Home.

I would like first to give you some impressions of the development of biochemistry up to that period. It has been my task to lecture on enzymes from 1920 onward; I can see now very clearly that until 1920 folk were trying to learn their enzymic kinetics and how to estimate them: in other words quite

elementary things about enzymes were not then really known. It must be remembered that in the early 1920's Willstater entered this field with all his energy and resources and experience gained in isolating pigments. As a result of work by himself and his collaborators, much new knowledge was obtained upon the means of separating proteases, lipases, and other enzymes, and in particular upon the systematic development of the study of adsorbents, including the famous C-gamma aluminium hydroxide. In spite of all his work it went less far than might have been expected, because at that time no one had the slightest evidence that an enzyme was a protein. Research on the biochemistry of muscle, in which I had kept an interest since the work with A. V. Hill, took on a new turn when Meyerhof began to work with KCl extracts of minced muscle; this work then proved that the changes which took place in such extracts were not merely bacterial as had been shown so clearly for so much of the earlier work. This began to open up an entirely new phase in our ideas of biochemistry, which together with the tissue slice technique of Warburg, has turned out to be very important indeed. About this time Hopkins excited us greatly by his brilliant work on SH groups and glutathione. In one way this set the stage for a large amount of work for 15 years in the Cambridge laboratory, because it focussed attention upon oxidation stages. There were several edges to the joke of some physiologists that Hopkins' laboratory lived during those years on methylene blue, and one of these edges unquestionably turned back upon the physiologist because this period has laid so much of our background knowledge of the dehydrogenases, now firmly welded into the corpus of biochemistry in its application to the whole reactions of the cell. It was a period of great expansion for Hopkins, and in fact the Cambridge laboratory became, as has been so well said, a Mecca for most people who wished to study in this field; the presence of D. Keilin in a laboratory nearby reinforced the strength of the attraction.

But my own life had led me to Oxford, at that time to a small embryonic laboratory which had been started by Benjamin Moore 18 months previously, and which it was my task to build up and to set on its feet. It is a healthy experience for someone who has worked in a large laboratory with many experts round him to go out and become part of an embryonic one, because he has so definitely to become independent. I had much help from two excellent colleagues in Oxford at that time, P. C. Raiment and G. L. Peskett, and soon afterwards from C. W. Carter, a life-long friend who is still in the Department. At the same time I took to Oxford Ernest Walker, who had joined me in work at Cambridge. The new environment was refreshing, quite apart from the valued contacts at my College with C. N. Hinshelwood[4] and others.[5] The chemists in Oxford were always most friendly, and I greatly enjoyed my contacts with the great organic chemist W. H. Perkin (Jr.). It was a considerable education for a young man to watch him

[4] Now Sir Cyril Hinselwood, President of the Royal Society.

[5] Including Sir Robert Robinson, past President of the Royal Society.

at work, tending with swift movement his several chemical reactions at once, as well as shooting out his short and pithy words of wisdom. He had an experimental outlook as definite as that of Barcroft; both hated argument when they could decide by experiment. Perkin's successor (Sir Robert Robinson), and Hinshelwood's election to the Chair of Physical Chemistry, continued the chemistry friendship. Professor J. A. Gunn, the pharmacologist, and G. Dreyer, the pathologist, were also helpful colleagues. At the same time I was looked at a little askance by some other folk and had it not been for the powerful, valuable, and disinterested friendship of Sir Charles Sherrington and of Sir Archibald Garrod, I think that my task would have been harder.

When we started we had the old premises devoted to physiological chemistry in which, latterly, W. Ramsden had taught. These were in the laboratory of physiology, and this was certainly all which it would have been possible for Sherrington to provide for an incoming biochemist. He had remarkably little room himself for work which was of the highest importance to neurology; one thinks for instance of the research at that time on the stretch reflex by Liddell and Sherrington. My own space was of course much constricted. At one time my personal room was an office, library, and research room! In regard to the development of the present Oxford Laboratory of Biochemistry, I may say at once that by the munificence of the Rockefeller Foundation (Dr. Alan Gregg and Dr. Pearce) a grant which was very large for those days came to me in 1925, which enabled me to build the new laboratory. At the same time it allowed Sherrington to have larger space for the development of his own research. I can never be sufficiently indebted to the Rockefeller Foundation, and I have wondered to this day how they could have had such confidence in a very young man to give him this large accommodation. Sir Archibald Garrod had much to do with this; he was a distinguished physician and Regius Professor of Medicine at Oxford, succeeding Osler; but he had biochemistry in his chromosomes as the son of Sir Alfred Garrod, one of the first to use micromethods for estimating uric acid in blood in his practice on gout. His son Archibald will always hold a niche in biochemistry for his book on *Inborn Errors of Metabolism*, precursor in a way of much of the mutant work on *Neurospora*. His interest then was all on the rare disease haematophorphyria congenita. To him we owe the text in the Hall of my Oxford laboratory:

> For at the first "wisdom" will walk with him by crooked ways, and bring fear and dread upon him, and torment him with her discipline, until she may trust him, and try him by her laws. Then will she return the straight way unto him, and comfort him, and show him her secrets

found when reading the lessons in Christchurch!

In 1936 the laboratory was slightly extended, at the time of the installation of a Svedberg ultracentrifuge (mainly for the work of J. St. L. Philpot), and for which a grant was obtained from the Royal Society. This is now

under the charge of A. G. Ogston. About this time, too, the Department received valuable help from the great Nuffield benefaction. Later on, after World War II, the laboratory became again extended by two huts, one of which was devoted to the microbiological work of D. D. Woods, who has recently become Professor of Microbiological Chemistry. A direct connection maintained with the hospital clinical biochemistry was also most valuable. It was organised through J. R. P. O'Brien, the Nuffield Biochemist, who was also on our staff.

No doubt there is much that could be said of all these 31 years in Oxford, when I had the good fortune to have in my laboratory many, as colleagues and pupils, who have gained distinction in the United Kingdom and the United States. We constantly tried to keep the students up to date in their work, and they were excellent for their teachers, because they did not accept facts lying down. They challenged them and that was good for everybody. I would think there might be danger for a research institute in just this fact: that the research workers are not kept up to date by the penetrating remarks of students. Otherwise the administrative details, important locally, are not of much historical interest. As time went on, like other Universities, we became more and more dependent upon government support, and the head of a department found it essential to supplement any funds which the University and Colleges possessed by grants for studentships and the like from the big research Councils. This is the fact as it stands today; one may grumble at it to some extent, but it is difficult to see how it can be changed. I still think that the government should place at the disposal of every professor, who can reasonably be trusted to develop it well, a fund which is there for himself to determine, without, as it were, having to take round the hat. I can see the administrative difficulties, but I think that the gain in freedom to the Professor's mind would be immense, and most of the ones whom I know are more responsible in the way in which they spend their funds even than the Committees which make the awards, and they must know the students better.

It must be remembered that the character of the scientific work in biochemistry was changing during this period. It had altered from the static biochemistry which was very necessary in the early days, but which was strictly limited. The idea that once a molecule entered "protoplasm" it ceased to obey the laws of chemistry, had completely disappeared from the minds of those working at the subject. Fletcher and Hopkins gave the final quietus to this idea of the "giant" molecule. Calcium was still calcium even when it got into living tissue! Hopkins' brilliant address to the British Association in 1913 crystallised dynamic biochemistry and the dynamic equilibrium as a conception, though it took some 30 years before a textbook on these lines appeared by one of his pupils.

We seem to have travelled very far from the old controversies, now hardly embers even. That of vitalism versus mechanism seems to me particularly dead at the moment, because most of us, I imagine, now feel that

the one thing which distinguishes the living system is the complexity of its organisation, and with the increasing interest in nucleic acid chemistry, some of us may believe that if this system is put together it will work. We do not now have to think that there must be some very low grade metabolism going on for things to be potentially alive, as we have the experiments of Parkes and colleagues in which mammals have actually recovered after freezing. Surely J. S. Haldane's plea in his philosophy arose from the intense and justified conviction that some of the more remote work on isolated nerve at that time was leading right away from the real problems of physiology, and that any research done must ultimately be orientated in outlook towards the whole animal. His classic work on respiration pointed the moral. In my opinion we cannot escape from the fact that biochemistry is essentially applied chemistry of a particular type. We may get things out of living matter which are of much interest to pure chemistry, and they have very often proved to be so; but there always comes for the biochemist then the philosophical question whether the chemical facts obtained are of significance to the going living system; if not they may be of little interest to biochemistry as I conceive it.

Turning back to the research, it was the dynamic outlook with its preoccupation with the behavior of small molecular substances in the cell which took folk in the 1920's further into the problems of enzyme action, problems which progressed much more intelligently after Sumner crystallised as a globulin the urease of jack bean, and therefore showed that two of the outstanding problems, that of the enzyme and of the protein, were in essence one. Together with the work on oxidation and the SH compounds, this oriented much of the research during the period of 10 to 15 years from 1920 in Cambridge, at a time when we were stirred by the main work of Warburg, Embden, and Meyerhof; based upon the isolated and purified enzyme entities this crystallised our ideas about the organisation of glycolysis. It was essential to pass through this stage before we could take the next jump in attempts to penetrate action of the enzymes inside the living cell; early in the 1930's I ventured to point out that we should soon be in the phase of what was best called "coordinative biochemistry," and tried to crystallise ideas with geographical notions of the cell, the cytoskeletal hypothesis, developed from preoccupations with surfaces. Biochemical research, taken as a whole over this period, will come to be recognised as one of the major intellectual achievements of all time. The revelation of nature as a chemist never fails to astonish. At present we are in the middle of this coordinative phase with attempts to understand the organised action of fragments isolated from inside cells, variously called particles, homogenates, microsomes as well as mitochondria, a term almost overworked just now. In all this the tracer studies (radioactive and otherwise) have now revealed a dynamism quite unpredictable before. With the electron microscope some of us may hope to witness for the first time what Keith Lucas used to call the

ideal histology of the future in which the biochemistry is integrated with the histological structure.

Going back to some of my own work in Oxford, carried out over this period with colleagues, I used to be accused of two things at the same time which were incompatible: on the one hand of working at only one subject, and encouraging all those round about to work only at the subject of thiamine; and on the other hand of dodging about far too much, of working on protozoa, on surfaces, etc. In fact, there was some plan behind it, because I always felt that to be a really proper biochemist one must have a broad background. Somehow or other three main interests became integrated: the interest in surfaces; the interest in nutrition with especial reference to thiamine, which led to much biochemical work on brain, and in the background, this pharmacological interest in the biochemistry of intoxications. When I started to work on the problem of the isolation of thiamine, very few seemed to be at work; when I finished some 10 years later, a very large number were concerned, so that the work we did on the isolation was very largely submerged in the international effort from other laboratories. But I always felt that at least for myself a very important aspect of vitamin B studies was the dramatic change in the polyneuritic pigeon, so-called, on dosing with thiamine, i.e. the change from convulsive opisthotonus to normality. The impact which these facts made upon myself was no more remarkable than that upon students. I always tried to show this experiment to my advanced classes, as I never had trouble afterwards in interesting them in nutrition. The facts spoke eloquently for themselves. That this was really so was brought home to me 35 years later by someone whom I met casually, and who reminded me that I had once shown him this in the laboratory, and he said that it made such an impression upon him that he had never forgotten it! It was easy to realise that something fundamental would be found out if these events could be understood, and this was what induced me to study much further this and the biochemical conditions of the brain. I have always preferred to work on some phenomenon of this type, because it seemed to me so much more certain that differences found in the biochemistry would be of significance *in vivo*. The research led in the end to the demonstration of the *in vivo-in vitro* relationship between thiamine deficiency and these convulsions, and to proof of the connection between thiamine in the animal and pyruvic acid metabolism. Lohmann & Schuster's finding that the cocarboxylase of yeast was thiamine pyrophosphate connected the function of thiamine in the microorganism with our animal work, and in 1939 Banga, Ochoa, and myself succeeded in proving that thiamine in the animal was active in pyruvate metabolism as cocarboxylase.

Work of this kind made me (and I think many other biochemists) quite convinced of the extreme importance of the connection between enzymes and toxicology.

The most important part of the proof was attributable to the fact that

the biochemical lesion in the brain in thiamine deficiency was reversible both *in vitro* and *in vivo*. Garrod considered that Inborn Errors were a result of a missing enzyme, and Warburg that the cancer cell had a damaged carbohydrate metabolism. In neither of these cases could the phenomenon be reversed.

We were convinced, but others were not; and when we came to work at the start of World War II on these lines at the arsenic problem (and that arsenic problem had a very long history for me, since World War I), it was extremely difficult to convince our scientific friends in cognate fields that anything of great importance could come out of an *in vitro* study of enzymes. I do not think that the folk concerned had really taken in the extreme significance of these few experiments on the pigeon brain. Those of us in the early 1930's[6] felt that we were working at what was in effect the proof of a new approach to pathology, that in future it might be possible to pick up the biochemical changes which I called "biochemical lesions" before they had become pronounced enough to reach the stage of pathological and histological damage. It was in this sense that we felt it to be a new approach to medicine, and one that was of course not likely to meet with rapid acceptance. The value of this approach was demonstrated when our group, and especially Stocken and Thompson, finally reached the substance 2,3-dimercaptopropanol (BAL) which could take off the arsenic group attached to the thiol substance, a research which attained its biochemical completion with the isolation of lipoic acid in the United States. I know that the experience for me in the nutritional field with the thiamine research was a very valuable one. Anybody who is inclined to think that biochemical or biological research is easy, or alternatively is inclined to accept rather glibly certain assumptions, might be recommended to turn to the nutritional field because it is an extraordinary and very gruelling training to learn not to accept anything at its face value. There is also a width of application of nutritional research to the whole world today, with its multiplying population, which in spite of the length of time that such researches take, gives a good deal of satisfaction. Taking the case of thiamine alone, its isolation and knowledge of its biochemistry means that there is no excuse today for anyone to suffer from beri-beri.

Since the war my own efforts have been largely devoted to an attempt to unravel the reactions given by the monofluorocarbon compounds. This work is very recent and is going on at the present time in the new laboratory, and I do not think there is anything very special that I need say about it which cannot be found in reviews, except that it was an intellectual comfort when it did not prove an exception to the idea that there was an enzyme involved, and that a new type of attack was found, which I have called a "lethal synthesis," of which there are now quite a few examples.

Taking a broad view of one's research efforts, it seems that there were

[6] Including Gavrilescu, Passmore, Meiklejohn, Sinclair, and Thompson, as well as Kinnersley and O'Brien.

three turning points, one when Barcroft and the Cambridge group diverted me into research, secondly when the war gases gave me an impetus from which most of my other researches may be said to have sprung, and lastly when I became a member of the laboratory of Gowland Hopkins, which led to the Chair in Oxford. It was the success of the logical attack upon the arsenical problems, and the challenge produced by the fluoroacetates in so far as at first they did not seem to fit into the picture, which was the starting point for our present researches on the fluorine compounds. I have not mentioned anything about the advance in techniques, as I have seen them through the years; but these are such common knowledge that they hardly merit space.

It has been said so often that a man's best ideas occur in the 20's or early in the early 30's that I think some remarks are wanted in relation to biochemistry. Though I am prepared to think that this may be true of mathematics and possibly of theoretical physics, I emphatically do not believe that it is true of a biological subject. The ideal biochemist should be trained in all those subjects which will enable him or her better to understand the working of the living cell by itself and in its integration with the other cells to form the whole physiological make-up of the animal. To do this, the biochemist should have a reasonable grasp of organic and physical chemistry as a grounding, of the static side of biochemistry and of what is known of the dynamic biochemistry, and also of physiology in its broadest sense. It is best that this should extend into microbiological biochemistry and into pathology as well as pharmacology. If there is time a medical qualification does not come amiss. The training is almost that of a devotée; still if undergone, the fun in a biochemical life well repays the effort. This I know is preaching to the converted! Biochemistry requires continuously a dual mode of thought. All this takes time, and I do not see how it can be done before the age of 40. It is quite good for time to be spent in small pieces of research in the different disciplines as this always gives the greatest insight into each. The general knowledge of technique and grasp should then enable much more important and fundamental thought into the biochemical workings of the cell. Hopkins had a large background before he devoted his whole time to biochemistry. I understand that Bach in his music worked on this general plan of training. In my humble way I have tried to do likewise. Of course it is true that not so much progress is made at first in this manner. There cannot be the same impressive array of papers devoted to one subject; but what is done later may be done with a wider base. One thing I have strenuously disagreed with all my life is the idea that unless some discipline like chemistry or physiology is learnt between the ages of 18 to 22, it can never be learnt afterwards. It is so often said and is one of those dangerous half truths. The reason that so many biologists do not appear able to learn chemistry later is that they are not really interested; they never feel that thrill at the appearance of crystals, or that satisfaction when facts fit an equation which the chemists proper have been trained to understand.

It might be thought that team work is the ideal solution, and it has often given, and will continue to give, excellent results. It is true also that no biochemist can advance without experts; one may instance infrared spectroscopy. Yet I find that I have the conviction that there should be someone in the team with a sufficiently wide grasp to understand what is going on as a whole. Fortunately, members of a team always educate one another.

One of the trends in the modern egalitarian state is to believe that all must be up to a certain scholarship level by a certain period. My own observation teaches me that this is quite wrong. Development takes place at different rates, and I have seen many slow starters do magnificently afterwards. It is an interesting commentary upon this that at one time among those who had fellowships at my Cambridge College, a large proportion did not come up to the College with Scholarships.

I would like to see advice to young men (and to the administrators concerned with finding them grants for research) that they should aim at results in the 40's and 50's, and not confine their horizon to what can be achieved in the 20's and 30's. Though I agree with what Mellanby wrote in last year's article, that the trend of modern biochemistry has been much away from biology into chemistry, I do not agree that this has been too much, and that it was not a necessary development. So often (as in the case of the BAL story) the ultimate solution will lie even for a practical pathological problem in somewhat apparently remote chemical detail, and this requires chemical "nous." What is wrong is not the pursuit of this, but the fact that those engaged upon it have not developed the width of outlook required to understand how their biochemistry fits in. The old Pasteur-Ludwig conflict is still with us in essence. Simplifications and generalisations continually come; pH is an instance. But forever as we dig deeper other facts emerge to defeat our generalisations. Of course it must surprise the physiologist to be told, as sometimes has happened, that the results of some highly artificial biochemical experiment can be applied directly to the living animal; in some cases this is just a failure to understand the complexity of life, and the cure for it is to embark upon some research in which the whole animal is used. This is only saying again that biochemists should have a real knowledge in addition to their own subject of chemistry and of physiology or some such cognate science. I am most glad to note that there is a recent trend in education of biochemists and physiologists to recognise these facts, so that the old gibe that biochemists were more "bio" than "chemists" is becoming as much out of date as that they only worked with slime!

Many of the remarks made in this note have been put better before me by the other distinguished "older statesmen" who have written. Nonetheless, it seemed best to put down convictions; their coincidence with those of others reinforces the points the more. There are some other general points: what is the ideal size of a laboratory? Surely that where the Professor can comfortably keep in touch with all; few of us could emulate Hopkins in his intensely intimate touch, but any Professor should know his folk. I do not

see how he can do this properly with commitments in the University and outside, if his numbers are much over 40. Unfortunately success attracts more workers (we should therefore not pray for it!) and though there is some gain with numbers, the loss on balance is greater. Sherrington's influence was wide, but he never had many in his laboratory at one time! Each pupil was intimately trained by a master; as has been said, one in a century. Each Professor works in his own way; but several could find more enjoyment if they could work occasionally at a bench. Many do not find time to do this because they feel that it is robbing their laboratory as a whole of time which should be spent in looking after the interests of those about them. I believe that this is a mistaken conscience, and that the gain to all concerned would be greater than the loss in administrative time; even this could be made up by help from elsewhere.

There have been some losses to offset the gains in comfort and equipment of laboratories at the present time. With the support from government sources and the altruistic finance of research by industry, no research worker should complain about his chances of getting good work done; it may well be that he does not have to struggle enough. But the very fact that things are easier has led to a more professional outlook on research and to the attraction of workers who would not have given their lives to this in the past, when we could all say that we were amateurs. With increasing numbers, it must be recognised that for various reasons it is not so easy to have free discussion of work with every visitor; in my young days we never hesitated to discuss our work with others, and felt that it usually did not bore them. In spite of this loss of freedom the brotherhood of science is still there; one has only to have a meal with all nationalities at the Zoological Station at Naples to realise what international cooperation could be if all had the outlook of the scientists. I feel sure that it is more real even than the international exchange between monasteries in the Middle Ages. With all the ease in getting technical equipment, we may lose sight of the fact that though the speed of reading and digestion of the literature has undoubtedly much increased, the speed of thought has not accelerated much. Thought always implies struggle, and this struggle may well be relieved by a small amount of time at glass blowing! The struggle is inevitable as science is a form of art, and the scientist must continually suffer the daily frustrations of the artist. Perhaps this is why one can learn so much from Joshua Reynolds' "Discussion on Art." Fortunately the informed public are beginning to understand a little better that they can only expect 5 per cent of research to be rewarding; but that this 5 per cent is transforming the world.

Of the future, surely this is bright. The subject is probably at the moment more active even than nuclear physics, so it cannot fail to be an exciting adventure. The groundwork is there now in biochemistry, and we are beginning to close up on the problem of applying this to the living cell: I would say beginning because there are still before us some years of effort in the understanding even of the biochemical behaviour of cell particles. From

present trends there should be accelerating and startling practical applications of biochemical knowledge, as well as the intellectual satisfaction of an increasing understanding of what life is about. As I once said in a lecture:

> Then at last shall we obtain a picture of the co-ordinative working of a system, the marvellous intricacy of which we can but dimly guess. For a rigid utilitarianism is not a final aim. The scientific artist portrays the harmony of nature. Poincaré expresses this when he speaks of the search for this special beauty, the sense of the harmony of the world. Only those who have studied them can appreciate the fugues of Bach, which may justly mirror the concurrent and harmonious actions of the living cell. Biochemistry is a study of Nature's counterpoint. Many have not seen its beauties, but they are there for those that will.

Acknowledgments.—I am grateful to several colleagues for discussing with me the lines which this article should take; and particularly I wish to thank my wife for help in the composition, and for her encouragement and support over many years.

BIOLOGICAL OXIDATIONS[1,2]

By Henry R. Mahler
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The topics of this review are among the most active in biochemistry From the pages of about twenty journals, some 1200 papers flooded the consciousness of this reviewer during the period covered,[3] and a selective process had to be applied.

In common with previous practice, the yardstick has been mainly the subjective one of personal bent and predilection. Many excellent contributions had to be omitted, and this review contains, inevitably, errors of commission and omission. The emphasis will be on mechanism of action on both chemical and metabolic levels. The author will proceed from a consideration of the mode of action of individual pyridinoprotein, flavoprotein, and cytochrome carriers to the more complex enzymatic forms; to processes of electron transport, oxidative phosphorylation, and metabolic cycles.

Pyridine Nucleotides and Pyridinoproteins

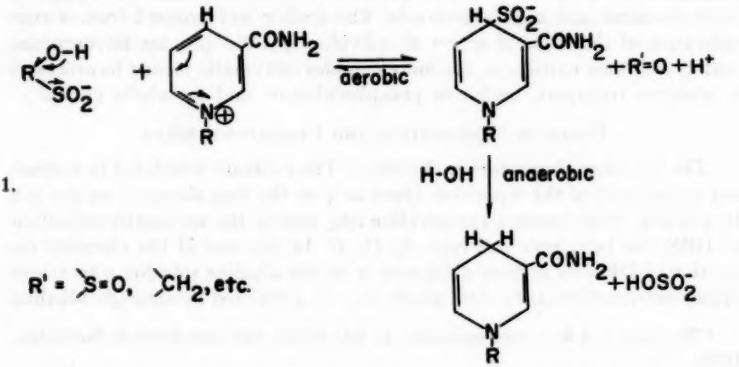
The reduction of pyridine nucleotides.—The evidence which led to a clear-cut assignment of the 4-position (*para* or γ to the ring nitrogen) as the site in which hydrogen enters the pyridine ring during the enzymatic reduction of DPN has been reviewed recently (1, 2). In the case of the chemical reduction of DPN by sodium dithionite in mildly alkaline solution a transient yellow intermediate is formed which may be stabilized by strongly alkaline

[1] The survey of literature pertaining to this review was completed in November, 1956.

[2] The following abbreviations are used in this chapter: ADP for adenosine-5′-pyrophosphate; ATP for adenosine-5′-triphosphate; BAL for British anti-Lewisite (2,3-dimercapto-1-propanol); CoA for coenzyme A; DPN or DPN^+ for oxidized diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; DPT for diphosphothiamine; E. for enzyme; EDTA for ethylene diamine tetraactic acid; ETF for electron-transferring flavoprotein; FAD for flavinadenine dinucleotide; $FADH_2$ for reduced FAD; FMN for riboflavin-5′-phosphate; GDP for guanosine-5′-pyrophosphate; GSH for reduced glutathione; GSSG for oxidized glutathione; GTP for guanosine-5′-triphosphate; K_{eq} for equilibrium constant; K_m for Michaelis constant; P_i for inorganic phosphate; PP_i for inorganic pyrophosphate; PPP_i for trimeta phosphate; TPN for oxidized triphosphopyridine nucleotide; and TPNH for reduced triphosphopyridine nucleotide.

[3] Four books have been published during 1956 which contain reviews or comprehensive contributions dealing with biological oxidations: *Advances in Enzymology Vol. XVII* (Interscience, New York, N. Y.); O. Gaebler, *Enzymes, Units of Biological Structure and Function* (Academic Press, New York, N. Y.); W. D. McElroy and B. Glass, *Inorganic Nitrogen Metabolism* (The Johns Hopkins University Press, Baltimore, Maryland); and D. E. Green, *Currents in Biochemical Research 1956* (Interscience, New York, N. Y.).

conditions (3). Dithionite reduction is the preferred chemical means for the preparation of enzymatically active DPNH, and it has frequently been regarded as a model system for dehydrogenase action (4, 5, 6). Since a free-radical structure for the yellow intermediate had been suggested (7, 8, 9), this problem may have considerable bearing on the more general one of enzymatic hydrogen transfer in reactions of this sort. Yarmolinsky & Colowick (10) have investigated the structure and function of the yellow intermediate and have identified it relatively unambiguously as the sulfinate adduct of DPN^+, analogous to other DPNH derivatives formed by the ionic addition of carbonyl reagents to the *p*-position of the nicotine ring in DPN^+ (11). Unlike these other, reversibly formed, adducts, on incubation under anaerobic conditions, the DPNH-sulfinate is irreversibly and almost quantitatively decomposed to DPNH according to Reaction 1.



1.

Perhaps Yarmolinsky & Colowick's contention is borne out most convincingly by their demonstration of the occurrence of Reaction 1 from left to right in the presence of organic sulfinates and from right to left in the presence of formaldehyde. In heavy water under moderately alkaline conditions only one of the two *para*-hydrogens of DPNH became labeled with deuterium. Thus Reaction 1 proceeds in two steps: ordinarily deuterium enters exclusively in the second step; only at very high pH, where enolization of the *para*-hydrogen in the adduct may be expected, is there exchange during its formation.

The nature of the yellow intermediate has also been investigated by Swallow (12). He has ruled out a free radical structure on (*a*) the basis of its resistance to further reduction by catalytic hydrogenation, x-irradiation in the presence of ethanol or on the surface of palladium foils (13), and (*b*) by means of paramagnetic susceptibility measurements. He also demonstrated an interaction of the intermediate with acetaldehyde and glycine, presumably by a reversal of Reaction 1.

If, on the other hand, DPN^+ is reduced by a reaction which definitely in-

volves reducing free radicals, as for instance by γ- or x-irradiation in an anaerobic aqueous medium in the presence of readily photolysed organic molecules, such as alcohols, etc. (13, 14), then a product is formed with properties quite dissimilar from those obtained on enzymic or dithionite reduction [Swallow (15)]. The compound can be shown to be a dihydro derivative of DPN, to be transformed to the same acid decomposition product, and to absorb at 340 mμ. The extinction coefficient at this wavelength is, however, only about one-half that of the usual form of DPNH, while that at 260 mμ is enhanced; the product is either totally (15) or largely [Barron *et al.* (14)] inert to enzymic reoxidation, and it lacks the characteristic fluorescence of DPNH. Similar properties have also been ascribed to a reduction product of DPN obtained on electrolytic reduction on either the dropping mercury or solid palladium, nickel, silver, or mercury electrodes [Ke (16)]. Reduction at platinum and lead electrodes gives a product which is partially reoxidizable by acetaldehyde in the presence of alcohol dehydrogenase, although no values comparing the rates of oxidation of the two types of products have been reported [Ke (17) (18)]. By use of the model compounds 1-propylnicotinamide iodide and 1-(2,6-dichlorobenzyl)-nicotinamide bromide respectively, Stein & Stiassny (19) and Wallenfels & Schüly (20) have demonstrated that two different crystalline isomeric reduction products may be obtained, depending on the reducing agent. Dithionite in both cases yielded a material with an absorption peak at 360 mμ corresponding to that at 340 mμ for DPNH, and identified as the *p*-isomer. x-Irradiation gives the other isomer with peaks at 350 and 266 mμ. Borohydride reduction yields a product reoxidizable enzymatically to an extent of approximately 50 per cent [Mathews & Conn (21)]. In the case of the model systems this led to approximately equimolar mixtures of the two reduction products. Thus, reagents which probably proceed by an ionic reduction mechanism appear to yield the enzymatically active *p*-isomer, while those which utilize a free radical path give rise to an inactive one, presumably one of the *o*-isomers. Borohydride which may be a donor of hydride ions as reducing species gives a mixture of the two isomers. It may be germane to note that Mauzerall & Westheimer (22) showed that only the *p*(4-)deutero-isomer of 1-benzyldihydronicotinamide was able to reduce malachite green in a direct hydrogen-transfer reaction analogous to the enzymatic one, but that neither of the two *o*-isomers (2-, or 6-) was able to do so. Similarly the *p*-isomer was able to reduce the free radical, diphenyl picryl hydrazyl. According to Wallenfels & Schüly, however, the *o*-isomer (either 2- or 6-, obtained by borohydride reduction) of their very similar compound, reduced the same acceptor even somewhat more rapidly than the *p*-isomer. In further investigations of nonenzymatic models for pyridine nucleotide action Abeles & Westheimer (23) showed that, although their model compound, 1-benzyl-1,4-dihydronicotinamide, did not reduce ordinary aldehydes and ketones, both it and DPNH were capable of reducing the more reactive thioketone, thiobenzophenone. By use of the 4-deutero compound and by conducting

the hydrogen transfer in heavy water it was demonstrated that this process was a direct one from reductant to oxidant, similar to the enzymatic reactions.

Other derivatives of pyridine nucleotides.—Interest has recently been shown in the addition compounds of pyridine nucleotides with various carbonyl reagents not only because of their intrinsic importance as structural analogues of DPNH, as analytical tools for the assay of DPN and as a means of explaining the inhibitory action of a wide variety of reagents, but also as possible devices and model systems for the study of the dehydrogenase reactions proper [Kaplan (11)]. The addition compounds prepared so far may be regarded formally as being formed according to Reaction 2. Some of their properties are summarized in Table I.

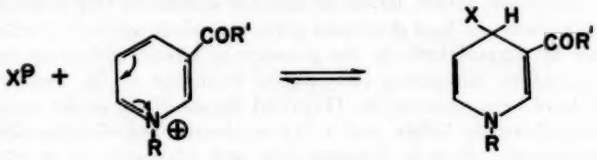
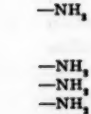
2. 

TABLE I

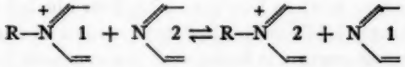
SOME DPNH DERIVATIVES AND ANALOGUES

R'	X—	pH	Other Component	λ_{max}	α_{mM}†	Reference
$-NH_2$	—H(DPNH)		—	340	6.3	
$-NH_2$	—OH	>13	—	340		(24)
$-NH_2$		9.8	—	340	~6	(25)
$-NH_2$	—CN	11	—	325	6.3	(26, 27)
$-NH_2$	$-SO_3H$	7–8	—	320	4.8	(26, 27, 28)
$-NH_2$	—NHOH	10.6	—	315	~6	(29)
$-NH_2$	$-SO_2^-$	~12	—	360	~3	(10)
$-NH_2$	—NHOH		liver alcohol dehydrogenase	300	5.8	(30, 31)
$-NH_2$	$-SO_3H$		heart malic dehydrogenase	320	4.8	(28)
$-NH_2$	—SH		liver lactic dehydrogenase	320	6.8	(32)
$-NH_2$	—		glyceraldehyde phosphate dehydrogenase	330 350	?	(33, 34)
$-CH_3$	—H (acetyl DPNH)		—	365	7.8	(35)
$-CH_3$	$-SO_3H$		—	340		(35)
$-CH_3$	—CN		—	340		(35)

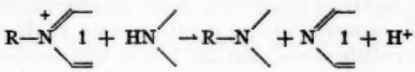
† Millimolar absorbancy index (approximate) in liters × millimoles^{-1} × cm.$^{-1}$

In general, the equilibrium of DPN with the various reagents shown is unfavorable for the formation of the addition compounds; the reactions involved require high concentrations of the various reactants and, except for

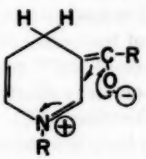
the bisulfite product, a high pH. In the presence of the specific enzymes indicated, the stability of some of the complexes is enhanced considerably, presumably due to a modification of the structure of enzyme bound, as compared to free, DPN. The nature of this modification is indicated by the experiments of Kaplan and his collaborators with the acetylpyridine and pyridine aldehyde analogues of DPN (35, 36) (R′=—CH_3 and —H). These derivatives were prepared by the action of a diphosphopyridine nucleotidase (from pig brain particles) in a reaction (Reaction 3) which involves exchange of the pyridine moieties.

3. 

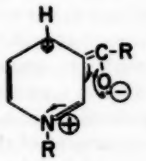
[A very similar enzyme from spleen has recently been solubilized and highly purified by Alivisatos & Woolley (37). Its range of activity even extends to reactions such as 3a (38).]

3a. 

The association constants of the acetylpyridine analogue with bisulfite, cyanide, and dihydroxyacteone are approximately one hundred times greater than the corresponding ones for DPN. Similarly the absorption maximum of its reduced form is at longer wavelength and its absorption more intense than DPNH. The E_0' of the couple, acetylpyridine/reduced acetylpyridine analogue, has been estimated as −0.248, compared with −0.320 v. for DPN /DPNH. The importance of considering structures such as I in any description of the reduced pyridine nucleotide molecules has been pointed out (35). Similarly the possibility of II as one of the resonance forms of oxidized pyridine nucleotides should not be disregarded.



I



II

The importance of the appropriate electrophilic substituent in the 3-position on the pyridine ring is borne out by Kaplan & Ciotti's finding that reduction by dithionite and ability to form addition products is observed when R′=—H, —CH_3, —NH_2—OC_2H_5, or —NHC_2H_5 (the last two compounds being inactive enzymatically) while the compounds with hydrogen, methyl, or hydrogen-methyl carbinyl as *m*-substituents were completely inert.

The sulfide derivative of liver lactic dehydrogenase·$(DPN)_2$, discovered

by Terayama & Vestling (32), is of interest because the sulfhydryl groups of pyridinoproteins may be directly concerned with coenzyme binding, especially in the case of glyceraldehyde phosphate dehydrogenase [Racker (34); Velick (39)]. The inhibitory effects of iodoacetate, hydroxylamine, and bisulfite on a series of seven pyridinoprotein enzymes have been compared by Pfleiderer *et al.* (28). Rather specific patterns emerged which suggest that interactions of coenzyme and apoenzyme strongly influence the chemical as well as the enzymatic reactivity of the pyridine nucleotide. Thus, its combination with yeast alcohol dehydrogenase is sensitive to —SH inhibitors and to hydroxylamine but inert to bisulfite; with liver alcohol dehydrogenase it is exceedingly strongly inhibited by hydroxylamine and much less efficiently by bisulfite, while iodoacetate is inert; with heart muscle lactic dehydrogenase, bisulfite is the most potent inhibitor and hydroxylamine barely shows any effect.

The interpretation of the inhibitory effects of these and other reagents on the enzymatic reactions involved is, however, complicated by their possible alternate role as metal-binding agents, in view of the recent discovery of Vallee and his collaborators that Zn^{++} is tightly bonded to the protein moiety of a number of pyridinoproteins (see 1). In general the number of Zn atoms per molecule of enzyme closely approximates the number of active sites postulated on the basis of the extent of coenzyme binding. Some elucidation of the role of the metal is provided by studies of Hoch & Vallee on the action of yeast alcohol dehydrogenase in the presence of the metal-binding agent 1,10-phenanthroline (40), which may react with the enzyme to form the inhibitory species: Enzyme·$[Zn\cdot(Inhibitor)_2]_4$. The inhibition is competitive to DPN, and DPN can protect against the action of the inhibitor if added prior to, but not after, the 1,10-phenanthroline. These observations have been interpreted as indicating a role of Zn in coenzyme-enzyme binding to yield the active enzyme species. If structures such as I and II are indeed of importance in the coenzymatic function of pyridine nucleotides then their contribution may well be enhanced by complexing of the negatively charged oxygen with the metal ion. Although the presence of Zn^{++} appears well documented in a number of instances(1, 41), the metal is absent from the highly purified lactic dehydrogenase [Terayama & Vestling (32)].

The structures of the addition compounds have been written to conform to that established for DPNH. These arguments have not so far been buttressed by structural chemical means and are largely based on similarities of optical properties, such as absorption spectra and fluorescence. The latter property has been used for the assay and study of the kinetics of pyridinoprotein dehydrogenases by means of fluorescence recorders with a sensitivity in excess of tenfold over the customary spectrophotometric techniques [Theorell *et al.* (42); Lowry *et al.* (43); Greengard (44)]. An inferential proof for the postulated structures comes from San Pietro who observed that only the hydrogen in the 4-position of the pyridine ring (i.e., alpha to the postulated position of the —CN group) in the DPN cyanide compound exchanged rapidly and completely with D_2O in the medium (45).

Krebs and his collaborators (46) have continued their studies on "DPNH-X" which is formed from DPNH either by the action of glyceraldehyde phosphate dehydrogenase in the presence of polyvalent anions [but not by other dehydrogenases (47)] or under the influence of a DPNH oxidase isolated from pig heart by Huennekens *et al.* (48). At first it was believed identical, on the basis of similarities in absorption spectra, with the modification product of DPNH formed by brief exposure to dilute acid (49). Reinvestigation disclosed, however, that the two compounds were not identical and that DPNH-X could be converted to the acid modification product (47). Analysis of DPNH-X shows the presence of nitrogen, phosphate, ribose, and adenylic acid in amounts and proportions expected for DPNH. Catalytic hydrogenation of both DPNH-X and the acid modification product gave only one readily hydrogenated double-bond compared to three for DPN and two for DPNH. The same workers have also isolated and partially purified an enzyme from yeast which reconverts DPNH-X to DPNH irreversibly according to Reaction 4 (50). Reaction 4 suggests that only a

4. $$\text{DPNH-X} + \text{ATP} \rightarrow \text{DPNH} + \text{ADP} + \text{P}_i$$

subtle difference in structure can obtain between DPNH and DPNH-X. From the available evidence, a hydration rather than an isomerization product is the more likely alternative.

Stereospecificity of the enzyme-catalyzed reductions.—As already indicated, pyridinoproteins can be regarded as falling into two classes depending on which side of the pyridine ring the addition of hydrogen occurs during the enzymatic reduction of DPN (1, 2). Lactic, malic, and alcohol dehydrogenase are representatives of what has been called the "α"-type, while the inducible β-hydroxysteroid dehydrogenase, isolated and recently highly purified from *Pseudomonas testosteronis* by Marcus & Talalay (51), is a representative of the "β"-type [Talalay *et al.*, (52)]. The latter class of enzymes which adds or abstracts hydrogen at the side opposite to that of the "α"-type also includes the transhydrogenase from *Pseudomonas*, and the two carbonyl compound dehydrogenases studied, viz., the glyceraldehyde phosphate dehydrogenase from yeast and mammalian glucose dehydrogenase [Levy *et al.* (53, 54)]. Two different flavoproteins capable of reoxidizing DPNH, namely milk xanthine oxidase and pig heart DPNH-cytochrome reductase, have been tested for their sterospecificity with this substrate [Vennesland (2); Drysdale & Cohn (55)]. The reductase is specific for DPNH of the β-configuration, while preliminary data with xanthine oxidase indicate a lack of specificity. These observations are of interest since the re-oxidation of protein-bound DPNH has been demonstrated, in the case of the glyceraldehyde phosphate dehydrogenase, by means of either pyruvate and lactate dehydrogenase, or by cytochrome reductase plus an appropriate electron acceptor (1). Apparently the two linked dehydrogenases may then function on opposite sides of the pyridine ring while the addition and removal of hydrogen in the presence of the dehydrogenase and the reductase would have to take place on the same side.

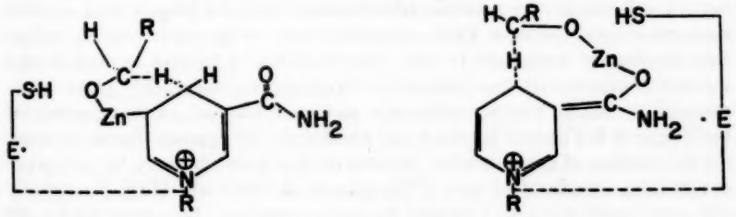
Linked reactions of this sort have been studied by Nygaard & Rutter (56) who found that the rate of oxidation of lactate by lactic-, of glutamate by glutamic-, and of alcohol by liver and yeast alcohol-dehydrogenases with DPN bound to the glyceraldehyde dehydrogenase were somewhat slower than those observed with free DPN, though of the same general order of magnitude. However, the reduction of pyruvate and acetaldehyde in the presence of their appropriate dehydrogenases was effected more rapidly by enzyme-bound than by free DPNH. The reaction mechanism in the case of the liver alcohol dehydrogenase, as determined from overall kinetics, appears to be identical for the free and bound forms of the pyridine nucleotides. The evidence led to the conclusion that free DPN (or DPNH) is definitely ruled out as a mobile shuttle in these linked reactions but that they may be catalyzed by a direct switching of the nucleotide from one enzyme protein to the next. Some spectrophotometric evidence for this contention was adduced.

Other effects on enzyme-coenzyme interactions.—Following earlier investigations on the old yellow enzyme, Theorell's group has recently considered the possible role of anions in pyridinoprotein catalysis. It may of course be anticipated from more general considerations that any ion such as a buffer, capable of interacting with the protein in the vicinity of the active site, may affect the binding of substrate and coenzyme and the reaction kinetics. This may be due to specific binding, or to nonspecific ionic strength effects as well as to changes of the charge-distribution of the protein molecule [Alberty (57, 58)]. Some of the specific effects may be in direct competition to the binding of substrate or coenzyme. Thus Theorell, Nygaard & Bonnichsen (42) found pronounced effects of anions on some of the velocity constants of the liver alcohol dehydrogenase reaction which were in some cases of opposite sign for the oxidized and reduced forms of the coenzyme-enzyme complex. Chloride, for instance, enhances the reaction velocity in the presence of large concentrations of both DPN and alcohol, but inhibits when the concentration of DPN is high and alcohol low. The activity of glucose-6-phosphate dehydrogenase, as found by Rutter & Rolander (59), is increased sixfold by low concentrations of certain anions while it is inhibited by higher concentrations. The magnitude of stimulation and inhibition, as well as the critical concentration when stimulation is changed to inhibition, depends not only on the nature and concentration of the anion but also on the concentration of the reactants. The order of effectiveness for anions is approximately $F^- > HPO_4^= > Cl^- > Br^- > SO_4^= > I^{-1} > SCN^-$. Cations also stimulate; their effect is generally related to the magnitude of their contribution to the ionic strength of the medium.

Another interesting approach is that of Shuster & Kaplan who studied the activity of the 3′-isomer of TPN formed from the naturally occurring 2′-isomer by acid isomerization (60). In general the results indicate that the only enzymes capable of interacting with the new pyridine nucleotides are those which do not show a strict specificity with respect to DPN or TPN.

Glucose-6-phosphate dehydrogenase of *Leuconostoc mesenteroides*, however, which can reduce both nucleotides, does not react with the 3′-isomer. The bacterial pyridine nucleotide transhydrogenase when acting on 3′-TPNH as compared to TPNH is much more strongly inhibited by P_i, and this inhibition is much more readily reversed by 2′-adenylate. The latter compound alone is an effective and competitive inhibitor of TPN for a variety of dehydrogenases specific for this nucleotide [Neufeld *et al.* (61)].

Properties, reaction kinetics, and mechanisms of highly purified pyridinoproteins.—The yeast alcohol dehydrogenase reaction has been reinvestigated by Nygaard & Theorell at 23°, in the pH range 6.0 to 7.15, by means of their sensitive fluorescence recording technique (62, 63). Within this pH range a more clearcut distinction can be made between the various possible mechanisms as determined from the relationships between the observed kinetic and equilibrium constants [Alberty (57, 58, 64)] than was possible at higher pH's used by previous investigators (1). The mechanism which best fits the data is one which postulates rapid and reversible binding of both reactants to the enzyme surface to form relatively long-lived ternary complexes (presumably E[DPN·alcohol]$_4$ and E[DPNH·aldehyde]$_4$) which are transformed one into the other by intramolecular hydrogen transfer in the rate-limiting reaction. At higher pH the additional restriction obtains that the extent of binding of substrate is independent of the presence of coenzyme and vice versa (65). Essentially similar conclusions were also reached by Mahler & Douglas (66) for the reaction catalyzed by the enzyme at pH 7.6. Deuterium-substituted DPNH and ethanol were used and the kinetics of the interconversion compared with those observed with hydrogen-containing substrates. The isotope effects on rate and Michaelis constants were interpreted in terms of a mechanism similar to that postulated for the Meerwein-Pondorff-Oppenauer equilibrium in organic chemistry. Thus an enzyme-linked transition state may have a structure corresponding to III or IV.



III IV

Suggestions along similar lines have also been made by Vallee (67) and by Wallenfels & Sund (68). Carbonyl binding of Zn has been suggested in the case of the liver enzyme by Theorell *et al.* (42). Wallenfels & Sund have also considered the role of sulfhydryl groups on the enzyme. The turnover num-

ber varies linearly from zero (when the number of free —SH groups is four) to a maximum at 36 free —SH groups. The four residual groups are believed to be involved in the binding of the metal while the remaining thiol residues function in holding reactants in a configuration sterically favorable for the reaction. From the pH dependence of the reaction kinetics, Sizer & Gierer (69) conclude that the proton abstracted from or added to the alcoholic hydroxyl group is rapidly transferred to and from an acidic group on the enzyme surface and thence only slowly equilibrated with the medium.

The investigations on the yeast enzyme appear to rule out an aspect of the mechanism established fairly conclusively for the corresponding liver enzyme, viz., the nonparticipation of ternary complexes in the rate-limiting step (42). It has been pointed out, however, that the kinetic data merely make unnecessary the postulation of such complexes but do not rule out their rapid formation and decomposition. Thus, Reaction 5a would be kinetically indistinguishable from sequence 5b.

5a. $$\text{E·DPN} + \text{alcohol} \underset{k^-}{\overset{k^+}{\rightleftharpoons}} \text{E·DPNH} + \text{aldehyde}$$

5b. $$\text{E·DPN} + \text{alcohol} \overset{\text{fast}}{\rightleftharpoons} \text{E·DPN·alcohol} \underset{k^-}{\overset{k^+}{\rightleftharpoons}} \text{E·DPNH·aldehyde} \overset{\text{fast}}{\rightleftharpoons} \text{E·DPNH} + \text{aldehyde}$$

Recently the formation of the enzyme-DPNH complexes for this enzyme has been demonstrated by fluorimetric experiments (Boyer & Theorell 70), confirming earlier work [Theorell & Bonnichsen (71)].

The mechanism of the heart muscle lactic dehydrogenase reaction has been re-examined by kinetic means by Hakala *et al.* (72). The turnover number for the reduction of pyruvate by DPNH at 25° in 0.05 *M* phosphate, pH 6.0, is 4.15×10^4 moles $\times$ min.$^{-1} \times$ 135,000 per gm. enzyme. Using Alberty's criteria of the possible relationships between kinetic and equilibrium constants, the most likely reaction sequence is one involving obligatory binding of coenzyme by the enzyme followed by the formation of a ternary complex with the substrate. Hydrogen transfer takes place intramolecularly within that complex in a manner analogous to that postulated by Nygaard & Theorell for the yeast alcohol dehydrogenase. Some evidence for the binding of coenzyme by the enzyme has been obtained by independent binding studies [Schwert (72); Chance & Neilands (73)]. Essentially the same conclusions with respect to mechanism have been reached for the liver lactic dehydrogenase by Terayama & Vestling (32). The heart enzyme has been known to be relatively resistant to a variety of common sulfhydryl reagents (74). Recently, however, it was shown to be rapidly inactivated by Ag^+, Hg^{++}, and I_2, and reactivated by cysteine. Seven to eight thiol groups per mole could be titrated. These results have been interpreted as indicating that the enzyme-bound thiols are accessible only to small ions or mole-

cules and thus may not be involved in the binding of the relatively large reactant molecules [Nygaard (75)].

The heart muscle malic dehydrogenase has been obtained in a highly purified form by Wolfe & Neilands (76) who estimate its molecular weight from sedimentation and diffusion measurements to be $40{,}000 \pm 5000$. The Michaelis constants and turnover numbers have been measured at various pH values; the enzyme contains seven sulfhydryl groups per molecule as determined by Boyer's technique. Apparently the enzyme can function suboptimally with a lower number of thiol groups, however, since enzymatic activity is not abolished until all the available sulfhydryl groups have been bound by *p*-chloromercuribenzoate.

The full details of Moyle & Dixon's (77, 78) procedure for the purification of isocitric dehydrogenase from pig heart have now appeared. The enzyme, estimated as 90 to 95 per cent pure by physico-chemical techniques, has a molecular weight of 69,000. Both activities, i.e., the reversible dehydrogenation of D-isocitrate with TPN and the reversible decarboxylation of oxalosuccinate in the presence of Mn^{++}, are catalyzed by the same protein, and probably involve only one (or two closely overlapping) sites on the enzyme. At pH 7.3 and 24° the turnover number for isocitrate is 350 (in the presence of 4.2×10^{-4} *M* Mn^{++}). The dehydrogenation of D-isocitrate does not require Mn^{++} *per se*, but, in its absence, under the usual conditions of the reaction, dissociation of the enzyme-oxalosuccinate complex is very slight. The complex accumulates, jams the active site, and no further reaction takes place unless it is removed by the second reaction. Similar considerations hold for the carboxylation of α-ketoglutarate in the absence of TPNH. An enzyme of very similar properties has been isolated from *Trypanosoma cruzi* [Agosin & Weinbach (79)].

The glyceraldehyde phosphate dehydrogenase continues to occupy the attention of a number of investigators. Accurate physicochemical determinations of an essentially pure enzyme from rabbit muscle by Fox & Dandliker have shown the molecular weight to be 137,000 by sedimentation and diffusion (80), 140,000 by light scattering (81). Spectrophotometric coenzyme-binding studies on an apoenzyme, freed of DPN by the charcoal column technique, indicate its capacity of binding one mole of DPN per 46,000 gm. of protein, or three moles per 138,000 (81). This is in good agreement with the results cited above and with previous data (83). Only about 70 per cent of the DPN so bound is capable of direct enzymatic reduction by the substrate. Warburg's objections to the implication of an acyl-enzyme intermediate in the reaction catalyzed by the yeast enzyme have been critically examined by Koeppe *et al.* (84). They have shown that the acyl enzyme complex is broken down by P_i at a rate at least as rapid as the overall reaction and that for this reason and because of detailed mechanistic considerations no valid obstacle remains to the participation of such an intermediate in the reaction. The acyl group is probably attached to the identical enzyme thiol groups both during its formation in the oxidative phase of the

reaction, and its decomposition during the phosphorolytic phase. Evidence is presented that the glutathione released by heating of the muscle enzyme does not appear to represent glutathione essential for enzymatic activity.

Other pyridinoprotein enzymes of special interest.—Pyridine nucleotide-glutathione reductases have been isolated and purified from baker's yeast and beef liver by Racker (85). In conjunction with different enzymes the glutathione so formed is capable of reducing disulfide compounds; a specific glutathione-homocysteine transhydrogenase has been isolated from liver (86).

6a. $$\text{substrate } H_2 + \begin{Bmatrix} TPN^+ \\ DPN^+ \end{Bmatrix} \rightleftharpoons \text{substrate} + \begin{Bmatrix} TPNH \\ DPNH \end{Bmatrix} + H^+$$

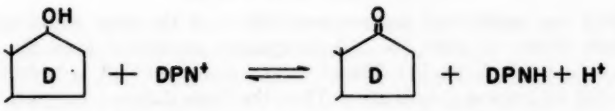
6b. $$\begin{Bmatrix} TPNH \\ DPNH \end{Bmatrix} + GSSG + H^+ \rightarrow 2GSH + \begin{Bmatrix} TPN^+ \\ DPN^+ \end{Bmatrix}$$

6c. $$2GSH + RS{-}SR \rightleftharpoons GSSG + 2RSH$$

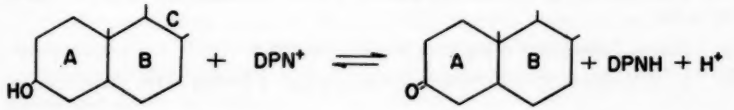
Systems of this sort (see Reactions 6a, 6b, and 6c) may be important in physiological processes where the controlled formation of a free thiol compound is required. One phenomenon of this kind is the essential role of reduced protein sulfhydryl groups in cell division of yeast [Nickerson & Falcone (87)]. Wolff & Kaplan (88) have demonstrated the presence in a variety of micro-organisms, and have purified from *Escherichia coli* B, an enzyme capable of the reversible interconversion of D-fructose-6-phosphate and D-mannitol-1-phosphate in the presence of DPNH and DPN (88). The enzyme appears to be quite specific for all reactants and to require one or more free —SH groups. At pH 7 the equilibrium of the reaction is strongly in favor of mannitol-phosphate formation.

Micrococcus lysodeikticus has been shown by Cohn (89) to contain two different malic dehydrogenases, both yielding oxalacetate as the product. One utilizes DPN as the coenzyme and appears quite similar to the mammalian enzyme, while the second does not seem to require the addition of any pyridine nucleotide, nor to reduce added pyridine nucleotide, on the basis of spectrophotometric data; it can be assayed by ferricyanide reduction. This enzyme bears some resemblance to one found by Scholefield in pigeon liver (90) and also to the "conjugated," i.e., tightly pyridine nucleotide-linked malic dehydrogenase of mammalian mitochondria [Huennekens (91)]. Two different DPN-requiring dehydrogenases which catalyze important reactions in the steroid series have been purified from *Pseudomonas testosteroni* by Talalay & Marcus (51, 92). One called the beta steroid dehydrogenase catalyzes Reactions 7a and 7b while a separate enzyme, the alpha dehydrogenase, is specific for reaction 7c.

Both enzymes are exceedingly unstable to the action of sulfhydryl reagents and heavy metal ions but are profoundly stabilized by DPN. The beta enzyme is strongly inhibited by estradiol-3,17β as well as by diethylstilbestrol and diethylhexestrol. While the bacterial enzymes are strictly DPN-specific, a very similar alpha-enzyme from mammalian liver has been reported by



7a.



7b.



7c.

Tomkins to utilize DPN and TPN with equal facility (93). An enzyme catalyzing the reoxidation of reduced lipoic acid by DPN has been isolated from yeast by Cutolo (94); cysteine is also oxidized while reduced glutathione is essentially inactive.

In the presence of soluble enzymes isolated from mitochondria Kun (95) has demonstrated the occurrence of the reactions designated 8a to 8e.

8a. d- or *meso*-tartrate + $DPN^+ \overset{Mg^{++}}{\rightleftharpoons}$ oxaloglycolate + DPNH + H^+

8b. oxaloglycolate $\rightleftharpoons$ dihydroxyfumarate (non-enzymatic)

8c. oxaloglycolate + $DPN^+ \rightleftharpoons$ diketosuccinate + DPNH + H^+

8d. oxaloglycolate $\xrightarrow{Mg^{++}}$ hydroxypyruvate + CO_2

8e. oxaloglycolate $\xrightarrow[EDTA]{Mg^{++}}$ glyoxylate + CO_2

Oxidation of Fatty Acids

Since the oxidation of fatty acids, recently elucidated on the enzymatic level, involves both pyridinoprotein and flavoprotein dehydrogenases, comment is appropriate at this point. A systematic nomenclature for the enzymes involved has recently been proposed by an international committee (96), and its recommendations will be followed in this review.

Pyridinoprotein systems.—Wakil has reported on the isolation of a DPN-specific D(−)-β-hydroxybutyryl dehydrogenase from beef liver mitochondria (97). The enzyme is very sensitive to thiol reagents and requires Co^{++} for full activity. It differs also in its stereospecificity from the DPN-requiring L(+)-β-hydroxyacyl dehydrogenase previously reported. By means of these two enzymes and the classical D(−)-β-hydroxybutyrate dehydrogen-

ase Wakil has established the stereospecificity of the liver enoylhydrase, previously shown to yield the L(+)-β-hydroxy derivative from the *trans* isomer, crotonyl CoA (98, 99). The *cis* form, isocrotonyl CoA, is hydrated to the D(−)-β-hydroxyacyl derivative. Thus the three different enzymes catalyze the sequence of interconversions and isomerizations shown in reactions 9a to 9e.

9a. *trans* ac-2-enoyl CoA + $H_2O \rightleftharpoons$ L-β-hydroxyacyl CoA — enoyl hydrase

9b. L-β-hydroxyacyl CoA + $DPN^+ \rightleftharpoons$ β-ketoacyl CoA + DPNH + H^+ — L-hydroxyacyl dehydrogenase

9c. β-ketoacyl CoA + DPNH + $H^+ \rightleftharpoons$ D-β-hydroxyacyl CoA + DPN^+ — D-hydroxyacyl dehydrogenase

9d. D-β-hydroxyacyl CoA $\rightleftharpoons$ *cis*-ac-2-enoyl CoA — enoyl hydrase

9e. (?)-β-hydroxyacyl CoA $\rightleftharpoons$ (?)-ac-3-enoyl CoA — enoyl hydrase

The situation is further complicated by the possible occurrence of an enzyme catalyzing the direct racemization of the D- and L-β-hydroxy forms [Stern *et al.* (100)] and Wakil's claim that some of his preparations catalyze reaction 9c without externally added pyridine nucleotide.

Stern *et al.* (101, 102) have also investigated the specificity of crotonase, the hydrase obtained in crystalline form from a soluble extract of beef liver. They interpret the results as indicating optical specificity in the dehydration reaction proper, in accord with reaction 9a, but believe that the enzyme also catalyzes reaction 10 and not 9d.

10. *cis*-ac-2-enoyl CoA → *trans*-ac-2-enoyl CoA

A puzzling feature is their observation that in reaction 9e vinylacetyl CoA forms exclusively the L(+)-hydroxy compound while *trans* hex-3-enoyl CoA forms the D,L racemate. In view of this statement, the portion of their proposed reaction scheme showing a reversible interconversion of 3-enoyl CoA and specifically L(+)-β-hydroxyacyl CoA hardly appears tenable. Stern *et al.* (103, 104, 105) have studied the reactions and enzymes involved in the further transformations of β-ketoacyl CoA derivatives, specifically the β-ketoacyl thiolase and β-ketoacyl-succinate thiophorase reactions.

Flavoprotein systems.—Previous reports show that more than one flavoprotein is involved in the reversible dehydrogenation of saturated acyl CoA derivatives (Reaction 11).

11. $R—CH_2CH_2COS—CoA + E—FAD \rightleftharpoons R—CH{=}CHCOS—CoA + E—FADH_2$

In addition to the previously described FAD-cuproprotein butyryl dehydrogenase (R varies from CH_3 to C_6H_{11}), two more enzymes catalyzing Reaction 11 have been obtained in highly purified form from mammalian liver by Crane *et al.* (106) and Hauge *et al.* (107). The two enzymes have different chain-length specificities (R: CH_3 to $C_{16}H_{33}$ and R: C_3H_7 to $>C_{16}H_{33}$). They are called the general acyl dehydrogenase and palmityl dehydrogenase respectively. They are flavoproteins of molecular weight 150,000 to 200,000

with two molecules of FAD per mole as prosthetic groups and containing small amounts (< <stoichiometric with flavin) of iron.

The various acyl dehydrogenases are incapable of direct interaction with most acceptors (except for *N*-methyl phenazonium sulfate and certain quinones) in the absence of yet another flavoprotein. This enzyme has been purified from liver by Crane & Beinert (108) and has been termed the electron-transferring flavoprotein, ETF. It is a relatively small protein of molecular weight ~50,000 with FAD (probably one mole per mole) as its prosthetic group. Its iron content is about one fifth that of flavin on a molar basis. The kinetics of the interaction of various fatty acyl CoA substrates plus their appropriate dehydrogenases on the one hand, and phenazine or ETF plus indophenol on the other, have been investigated by Hauge (109).

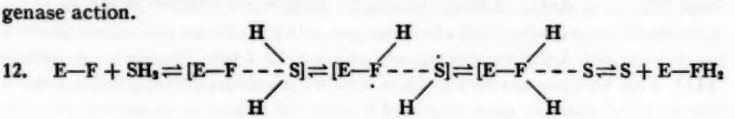
Flavoproteins

Semiquinone formation and the mechanism of action of flavoproteins.— Beinert has continued his study of anomalous spectral changes induced by addition of substrate to the various acyl dehydrogenases. At first, the apparently incomplete reduction of the flavin peak and the broad band appearing in the region 500 to 650 mμ (Λ_{max} at 570 mμ) was ascribed to the formation of a stable enzyme-substrate, or enzyme-product complex (110). A similar interpretation had also been placed by Dolin on analogous observations with the DPNH-peroxidase flavoprotein from *Streptococcus faecalis* (111). This became untenable when Beinert discovered that similar absorption spectral changes were obtained during the course of chemical reduction of pure solutions of FMN and FAD as well as during the reoxidation by O_2 of the reduced flavin nucleotides (112). The maximal concentration of the intermediate, presumed to be a semiquinoid free radical, was obtained at half-reduction. In acid solution the free radical is the red form previously described for riboflavin (113), characterized by a shift of the base of the flavin peak at the longer wavelength from about 500 mμ to 550 mμ, with a maximum for the difference spectrum between oxidized and reduced form at 503 mμ. In the pH range from 2 to 7 the intermediate has an absorption maximum at 565 mμ. There is also formed another intermediate with a strong absorption in the near infrared (830 mμ in acid, 880 mμ at pH 6.1, 1010 mμ at pH 8.9, and 770 mμ at pH 12.0 for FMN; with FAD the spectrum is shifted slightly to higher wavelengths). By the use of the criteria of concentration and temperature dependence enunciated by Michaelis (114), this peak was assigned to a dimeric (possibly quinhydrone-like) form of the free radical, which is formed from the monomer in a relatively slow reaction. Free radical formation in acid solution was also confirmed directly by microwave spectrocopy.

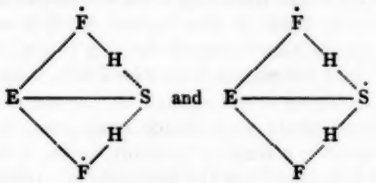
The spectrum of the acyl dehydrogenase during chemical reduction and reoxidation indicates that the characteristic bands of monomer and dimer, just described, made their appearance during chemical as well as during enzymatic reduction, and also when reduced enzyme is treated with oxidized

substrate (115, 116). Investigation of two other flavoproteins, viz. the L-amino acid oxidase from snake venom and the old yellow enzyme from yeast, both FMN enzymes, indicate the occurrence of similar intermediates during reduction with dithionite or substrate but to a much smaller extent than with the acyl dehydrogenases. Assuming a millimolar absorbancy index (α_{mM}) of 3.3 liters $\times$ millimoles^{-1} $\times$ cm.$^{-1}$ for the peak of the intermediate at 565 mμ, the maximum amount of intermediate obtainable experimentally in equilibrium with other forms corresponds to approximately >80 per cent for the acyl dehydrogenase, 30 per cent for the L-amino acid oxidase, 12 per cent for the old yellow enzyme and 6 per cent for free FMN or FAD. The semiquinones are formed and decomposed rapidly enough to permit their functioning as intermediates kinetically. A red semiquinone intermediate in the case of the old yellow enzyme had previously been reported by Haas, but its spectral characteristics appear different from those observed by Beinert, perhaps due to differences in technique (117). The DPNH and TPNH cytochrome reductases from mammalian heart and liver when reduced by their substrates show characteristic absorption in the 550 to 570 mμ region (118a, 118b).

Beinert has proposed a scheme similar to reaction 12 for acyl dehydrogenase action.



Transfer of electrons to ETF probably proceeds by way of the intermediate complex, which in this particular case is favored in the equilibrium reactions shown, from either direction. This extreme stability of a free radical intermediate is remarkable but may perhaps be accounted for by the fact that the enzymes in question contain two FAD molecules per molecule of enzyme. Assuming steric rigidity, tight electronic coupling and strong orbital overlap within the complex the two structures shown by Beinert viz.



may be equivalent and thus contribute to the stability of the intermediate. In the case of ordinary flavoproteins, additional stabilization can only come from flavin di-radical contributions. The importance of structures of this sort in the metalloflavoprotein case have already been pointed out (119, 120).

The occurrence of semiquinone forms during the reduction of flavin nucleotides is also borne out by the potentiometric titration data of Lowe &

Clark (121) who observed semi-quinone (monomer and dimer) formation of the order of 10 to 20 per cent in the region of half-reduction for both FMN and FAD at 10^{-3} *M*, and neutrality (121). Their empirical expression for the variation of the midpoint potential with hydrogen ion concentration is indicated in reaction 13.

13. $$E_M = 0.187 - 0.0601\ \text{pH} + 0.03005 \log \frac{2 \times 10^{-7} + [H^+]}{4 \times 10^{-11} + [H^+]}$$

Within the experimental error, values for FAD and FMN are identical; the calculated value of E_0' is -0.219 v.

Weber, Lenhoff & Kaplan (122) have studied the ability of selected flavoproteins to reduce an Fe^{+++} complex system containing citrate and 9,10-phenanthroline. At an iron concentration of approximately 10^{-3} *M* the relative rates of oxidation of reduced pyridine nucleotide by iron and 2,6-dichlorophenolindophenol were of the order of 1:5 to 10 respectively. The enzymes used were purified heart muscle diaphorase, liver TPNH cytochrome reductase and a crude extract of *Pseudomonas fluorescens* catalyzing the dehydrogenation of DPNH. The size and nature of the sample of enzymes tested for their iron-reducing ability was restricted, and they found that D-amino acid oxidase, a typical metal-free flavoprotein, reduced Fe^{+++} very slowly. The claims of Weber *et al.* (123) seem therefore premature that iron reduction (and the associated nonezymatic cytochrome-*c* reduction at relatively high iron concentration) is a general property of flavoproteins. Their studies do bear out the fact, however, that flavin nucleotides are capable of interaction with Fe^{+++} complexes and may explain the interaction of these enzymes with hemoprotein acceptors, particularly when enhanced by structural and functional juxtaposition of iron and flavin in enzymes capable of binding both these prosthetic group moieties (i.e., the ferroflavoproteins and proteins derived therefrom). Weber & Kaplan (124) also demonstrated that several flavoproteins, responsible for the dehydrogenation of reduced pyridine nucleotides by different acceptors, are capable of catalyzing the transhydrogenation of their specific substrates to the corresponding 3-acetyl pyridine analogue (see Reaction 14).

14. $$\left\{\begin{matrix}\text{DPNH}\\ \text{TPNH}\end{matrix}\right\} + \left\{\begin{matrix}\text{"acetylpyridine DPN}^+\text{"}\\ \text{"acetylpyridine TPN}^+\text{"}\end{matrix}\right\} \rightarrow \left\{\begin{matrix}\text{DPN}^+\\ \text{TPN}^+\end{matrix}\right\} + \left\{\begin{matrix}\text{"acetylpyridine DPNH"}\\ \text{"acetylpyridine TPNH"}\end{matrix}\right\}$$

Among the enzymes tested were pig heart diaphorase, pig heart DPNH-cytochrome reductase, *Neurospora* TPNH-nitrate reductase, milk xanthine oxidase, liver TPNH-cytochrome reductase and *Clostridium kluyveri* DPNH-oxidase. Although the oxido-reductions catalyzed by these enzymes appeared to be sensitive to *p*-chloromercuribenzoate, the transhydrogenations catalyzed by nitrate reductase, the *C. kluyveri* enzyme, and xanthine oxidase were unaffected by the inhibitor. Preliminary evidence indicates that FAD is not necessary and that transfer of hydrogen or electrons to

and from the protein may take place. The report by Drysdale & Cohn (55) that a sterospecific deuterium exchange from D_2O into DPNH occurs in the presence of DPNH cytochrome reductase, as well as with intact mitochondria, indicates that, at least in this case, the reaction involves direct hydrogen transfer to and from some group on the enzyme (which may or may not be the flavin nucleotide) capable of rapid equilibration of its protons with the medium. Thus reaction 14 may involve the steps shown in reaction 14a

14a. $$PNH + E + H^+ \rightleftharpoons PN^+ + E\begin{matrix}H\\ \\H\end{matrix} \underset{\text{very fast}}{\rightleftharpoons} E\begin{matrix}H\\ \\-\end{matrix} + H^+ \text{ etc.}$$

Reaction 14a is probably a prerequisite for the ordinary, oxidative functions of the flavoproteins, as indicated by Drysdale & Cohn's report that the oxidation of DPNH shows the same "β"-stereo specificity as the exchange. DPNH oxidation by xanthine oxidase, according to Vennesland, is nonstereospecific as is the oxidation of dideutero succinate by succinic dehydrogenase, [Englard & Colowick (125)]. Yet strong indications of direct hydrogen transfer from deuteroacetaldehyde to DPN in a reaction also catalyzed by xanthine oxidase were obtained by Vennesland (2). These direct transhydrogenation reactions seem mechanistically related to the ordinary reactions (presumably ionic) involved in pyridinoprotein catalysis and, if flavin is implicated, the general applicability of the free radical mechanism explicit in reaction 12 must be regarded with reservations.

Studies on isolated flavo- and metalloflavoproteins.—Nygaard & Theorell (126) have continued their studies on the interactions of the moieties of the old yellow enzyme by means of sensitive fluorometric and spectrophotometric techniques. Specific acetylation of a small fraction of the 60 primary amino groups of the apoenzyme strongly decreased the rate of resynthesis of the holoenzyme from its components, while the rate of dissociation was increased. In the presence of excess FMN, however, one mole of coenzyme could still be bound by the protein. The essentiality of free amino groups for tight coenzyme-apoenzyme association was also shown by means of other reagents, e.g., formaldehyde. Similar results were obtained with respect to tyrosyl residues: iodination of a small fraction of the total of 24 groups strongly decreased the association- and increased the dissociation-velocity. Both the iodinated and the acetylated apoenzyme appeared homogeneous in the ultracentrifuge with practically no change in sedimentation constant compared with the unmodified protein. FAD, riboflavin diphosphate, and atabrine, a common flavoprotein inhibitor, all appear to form loose complexes with the apoenzyme.

A potentially useful method for the study of flavoprotein systems has been introduced by Egami & Yagi (127). They showed that the D-amino acid oxidase was inhibited by adenosine monosulfate and riboflavin monosulfate with K_I values of 3×10^{-3} M and 3×10^{-5} M respectively. The former

is probably a competitive inhibitor of the adenylic, and the latter of the FMN moiety, of FAD. In the presence of both inhibitors the rate is depressed beyond that calculated for independent binding of the two compounds to the protein.

Xanthine oxidase continues to occupy the attention of many investigators. The properties of their crystalline preparation have now been reported by Avis *et al.* (128, 129). The best preparation appeared to contain more than 90 per cent of a single component by electrophoretic, sedimentation, and solubility analysis experiments. This component has an iso-electric point of 5.3 to 5.4, and a molecular weight ~290,000, indicating the presence of two or more FAD moles per mole of enzyme. In spite of this physicochemical evidence for homogeneity, enzymatic activity determinations led the authors to conclude that their preparation contained mixtures of two or more of the following species: the fully active enzyme, an "inactive" enzyme with a modified protein moiety, possibly capable of catalyzing DPNH-dehydrogenation, molybdenum-poor flavoprotein molecules derived from the inactive molecule and molybdenum-deficient molecules with a modified protein moiety. The last two are of unknown catalytic potency. The ratio of cofactors in the crystalline specimens was on the average 2:2 (actual values 1.3 to 1.5):8 for FAD:Mo:Fe. Iron and flavin seemed securely bound, whereas molybdenum was quite labile. The correlation of molybdenum content with enzymatic activity in a variety of test systems appears to be quite inconsistent. Bergel *et al.* (130) have recently reported on stabilization of the active form of the enzyme by salicylate, which should help in unravelling the tangled skein of evidence on this enzyme [for a recent review see De Renzo (131)]. Weber *et al.* (122) have confirmed earlier observations of Horecker & Heppel (132) on the oxygen requirement in the reduction of cytochrome-*c* by the enzyme. They have extended these observations to a relatively slow reduction of Fe^{+++} complexes catalyzed by xanthine oxidase and have explained them on the basis of formation of H_2O_2 in the aerobic reaction, followed by nonenzymatic reduction of the acceptor by the peroxide. However, neither Morell (133) nor Mackler *et al.* (134) have confirmed this oxygen dependence, with their enzyme preparations.

Glenn & Crane (135) have investigated the stimulation of the acetaldehyde-cytochrome-*c* reaction in the presence of the molybdoflavoprotein, aldehyde oxidase, by silicomolybdate complexes. The most active species had an Mo:Si ratio of 10:1. Phosphate could substitute for silicate in the complex but at decreased efficiency. Cytochrome-*c* reduction was shown to occur via an enzymatic reduction of the heteropolyanion followed by nonenzymatic reduction of the cytochrome acceptor. Thus this reaction provides a useful model for the simultaneous requirement of Mo^{+6} and phosphate during the reduction of cytochrome-*c* catalyzed by xanthine and aldehyde oxidases (134, 135, 136). Doisy *et al.* (137) have shown that this reaction is also stimulated by pre-incubation of xanthine oxidase with 9,10-phenanthroline or by addition of Fe-phenanthroline complexes. Their re-

sults on the action of various inhibitors on purified xanthine dehydrogenases and oxidases from chicken liver, rat liver, and milk indicate that pteridylaldehyde and cyanide may block dehydrogenation of purines and aldehyde dehydrogenase activity without blocking the corresponding reaction with DPNH. Similarly, Weber & Kaplan (124) observed that the reduction of a variety of electron acceptors in the presence of the enzyme is sensitive to *p*-chloromercuribenzoate when hypoxanthine is the substrate, but is unaffected with DPNH as the electron donor. It is apparent that the dehydrogenation of DPNH on the one hand and of purines and aldehydes on the other is catalyzed by separate sites on the enzyme molecule. When the complex nature of the hydrogen and electron transfers mediated by the enzymes is considered, the evidence cited does not necessarily militate against the involvement of FAD as the primary hydrogen acceptor at both sites. The cyanide block, as suggested previously, may be exerted subsequently in an intramolecular electron transfer at some metal site(s). [Mahler (120), p. 240].

Hofstee (138) has investigated the phenomenon of substrate inhibition of the enzyme with xanthine. Dietrich has discovered an inhibitory effect of hydroxylamine on the activity of both xanthine and D-amino acid oxidase (139). This inhibition may be due to the formation of an FAD-hydroxylamine complex similar to the one with riboflavin also described by this investigator (140).

Singer, Kearney, and their collaborators (141) have continued their studies on soluble, highly purified succinic dehydrogenases. Their most recent investigations indicate a molecular weight of approximately 200,000 for the enzyme from mammalian heart muscle, with one flavin molecule and four iron atoms as prosthetic group components (142). On aging this enzyme can be transformed to a two-iron form, showing one half the activity of the more complete type in the phenazine methonium sulfate assay. The flavin prosthetic group is bound very tightly to the protein, presumably by covalent amide bonds since it can be liberated only by proteolytic digestion. When isolated in this manner several different flavin-containing components can be identified on paper chromatograms. The predominant component is similar to FAD on analysis but differs in fluorescence intensity, R_F values, growth factor activity, and coenzyme potency in the D-amino acid oxidase test. Thus the flavin may be FAD with some portion of the original peptide sequence of the holoenzyme still attached. A strikingly similar flavin-adenine dinucleotide had previously been described as the prosthetic group of the ferroflavoprotein, DPNH-cytochrome reductase (143). Boukhine (144) has reported on the occurrence of a very tightly bound form of riboflavin in tissue associated with succinic dehydrogenase activity. An enzyme preparation almost identical in properties with those just described has also been reported independently by Wang *et al.* (145). A highly purified succinic dehydrogenase similar in all essential properties to the mammalian enzymes has now been isolated from yeast granules (146). The K_I for malonate with

the yeast enzyme is 1×10^{-5} *M* vs. 1×10^{-3} *M* for the heart enzyme, and it is inhibited rather than stimulated by 9,10-phenanthroline. Per gm. atom of iron, the heart enzyme is more active although the K_M for succinate is 1×10^{-3} *M* for each. Both preparations are capable of effecting the hydrogenation of fumarate either stoichiometrically, as demonstrated by the reoxidation of reduced prosthetic flavin, or catalytically, with leucosafranine dyes as reductants (147). The ready reversibility of the dehydrogenation of succinate catalyzed by these enzymes casts serious doubts on the separate existence of a fumaric hydrogenase [Fischer *et al.* (148)]. A particulate enzyme from heart muscle catalyzes a slow anaerobic exchange of deuterium from D_2O into succinate as shown by Englard & Colowick (125). This exchange is stimulated fifteenfold by fumarate and thus is probably a reflection of the reversibility of the reaction catalyzed by the enzyme. These results are in essential agreement with those of Erlenmeyer *et al.* (149), but contrary to those of Weinman *et al.* who obtained an exchange so rapid as to suggest labilization of protons as a requirement of the forward reaction, and to those of Geib & Bonhoeffer (150) on the other, who observed no exchange at all.

A soluble DPNH cytochrome reductase has been isolated and purified from the beef heart electron transport particle by De Bernard (151). It is a ferroflavoprotein similar to the one isolated previously from pig heart but it contains only two (instead of four) iron atoms and free FAD as prosthetic group elements. A highly purified hydrogenase has been obtained from *Desulfovibrio desulfuricans* by Sadana & Jagannathan (152). The enzyme requires Fe^{++} or Fe^{+++} for activity; it is capable of reducing methylene blue and benzyl viologen with molecular hydrogen at a rate of 2000 moles $\times$ min.$^{-1}$ $\times$ mg. N^{-1}. FAD, FMN, or riboflavin are reduced at 0.01 of this rate while DPN, TPN, sulfate, and ferricyanide are unaffected. The enzyme is sensitive to cyanide and sulfhydryl agents. The reduction by hydrogenase of a heat-stable cytochrome component also present in extracts of this organism can be demonstrated. The reduced cytochrome can be re-oxidized by O_2, methylene blue, or FMN.

Isolated, Purified Cytochromes

The previous scarcity of entities of this class, except for mammalian cytochrome-*c*, has now been replaced by a tidal wave. Not only bacterial systems, but even the recalcitrant particle bound hemoproteins of vertebrates and plants are being detached, isolated, and purified by methods of varying severity. What, if any, changes in their vital enzymatic properties occur only future investigations will disclose. There is no correlation for cytochromes of similar spectroscopic properties between their enzymatic, chemical, and physical properties and this makes imperative a revision of cytochrome nomenclature along more systematic lines than the presently used "cytochrome x_n" system. A suggestion to use the enzyme source and location of the alpha band as the keys in nomenclature was made a decade ago by Scarisbrick

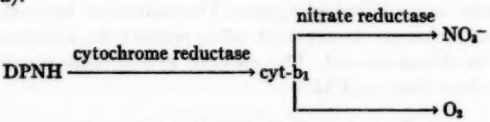
(153). It has not found wide acceptance, perhaps because, although precise, it is also somewhat cumbersome.

Cytochromes-"a" (*cytochrome oxidases*).—No report has as yet appeared claiming isolation or purification of a cytochrome of this type in the absence of bile salts or other solubilizing agents. Thus the molecular properties remain obscure. The oxidation of mammalian cytochrome-*c* by purified cytochrome oxidase preparations has been the subject of two recent studies.

15. $$\text{cyt-}c(Fe^{++}) + \text{cyt-}a(Fe^{+++}) \rightleftharpoons \text{cyt-}c(Fe^{+++}) + \text{cyt-}a(Fe^{++})$$

Wainio (154) has investigated the stoichiometry of reaction 15. He has estimated the ΔF° at $+729 \pm \sim 400$ cal. at 25°; the E_0' for cytochrome-*a* at 0.285 ± 016 v. (at 25°); and its differential (reduced-oxidized) millimolar absorbency index at 605 mμ as 7.3 liters per gm. atom Fe per cm. The kinetics of the reaction was found by Smith & Conrad (155) to be first-order with respect to cytochrome-*c* (Fe^{++}) over a wide range. The value of the first order rate constant, however, proved to be an inverse function of the total concentration of cytochrome-*c* present. Two new methods for following the catalytic cytochrome oxidase reaction have been described: Straus (156) has used the formation of a red quinone diimmonium pigment from dimethyl-*p*-phenylenediamine, while Longmuir & Clarke (157) determine the reduction of oxygen polarographically by means of a rotating gold electrode.

Cytochromes-"b."—A relatively low potential-cytochrome-*b*, tentatively identified as cytochrome-b_1 with Λ_{max} in the reduced state at 559, 528, and 426 mμ has been purified by Vernon from extracts of *Micrococcus denitrificans* and *Pseudomonas denitrificans* (158). Its prosthetic group was shown to be protoheme. Anaerobically the cytochrome can link DPNH but not succinate oxidation to nitrate reduction in the presence of the appropriate enzymes. Aerobically it is rapidly oxidizable in a cyanide-insensitive reaction (see Reaction 16a).

16a.


A very similar cytochrome (Λ_{max} at 558, 425 mμ) has also been isolated from an unidentified nitrate-reducing *Pseudomonad*.

Further observations on their yeast lactic dehydrogenase, previously identified as an FMN-cytochrome-b_2-containing enzyme (1), have now been published by Boeri & Tosi (160). In addition to the two moieties indicated (one mole each per 230,000 gm. protein) the enzyme also contains 8 gm.-atoms of Fe. The metal ion is not detached by dialysis against iron-chelating agents such as 9,10-phenanthroline. In the presence of lactate, an enzymatically active red complex, presumably [enzyme·(Fe^{++}·phenanthroline)$_3$] is formed. This suggests that the intramolecular flow of electrons shown in scheme 16b corresponds to that postulated for other ferroflavoproteins.

16b. $$\text{lactate} \rightarrow [\text{FMN} \rightarrow \text{Fe} \rightarrow \text{cyt-}b_2] \rightarrow \text{acceptor}$$

However, Chance *et al.* (161) who have studied the kinetics of these interactions have been unable to obtain spectrophotometric evidence for the stoichiometric reduction of any non-heme Fe^{+++} by lactate. The reaction catalyzed by the enzyme has been shown to be reversible, with $FMNH_2$ and pyruvate acting as reductant and oxidant respectively. The turnover numbers (moles lactate oxidized $\times$ $min.^{-1}$ $\times$ $moles\ enzyme^{-1}$) for different acceptors are 14000, 7000, 7000, and 2200 for quinone, cytochrome-*c*, ferricyanide, and methylene blue respectively. Thus this preparation appears to have higher activity with cytochrome-*c* than that crystallized by Appleby & Morton (162).

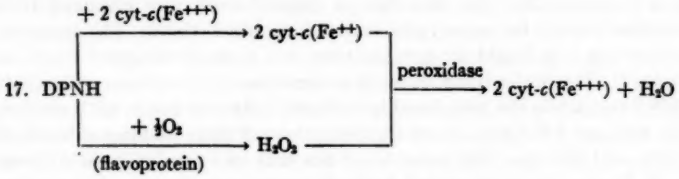
Publications by Strittmatter & Velick (163, 164, 165) concerning the enzymatic and chemical properties of the cytochrome obtained from microsomes of mammalian liver (cytochrome-b_5 or *m*) were discussed *in extenso* last year. The cytochrome, in the presence of a specific metallo(?)-flavoprotein reductase is capable of linking oxidation of DPNH to reduction of cytochrome-*c*, a cytochrome known to be specifically contained in mitochrondia and absent from liver microsomes. Its oxidation rate in air is slow, and thus the nature of the cyanide- and antimycin A-insensitive DPNH oxidation mediated by cytochromes of this type remains as obscure as ever. A somewhat purified preparation of the same cytochrome has also been reported by Garfinkel (166).

Cytochromes-"c."—It is no longer necessary to maintain a colony of King penguins if crystalline cytochrome-*c* is of the essence. Bodo's success (167) has now been duplicated by Hagihara *et al.* with the crystallization of the cytochromes from yeast, pig, and beef heart (168, 169). Comparison of the three crystalline preparations with a chromatographically purified heart muscle preparation (170) disclosed that all four hemoproteins, compared on the basis of their absorbancy at 550 mμ, showed equal activity in the cytochrome oxidase and succinoxidase assay. Spectroscopically the crystalline beef and yeast cytochromes show the following relative absorbancies: 1.00; 0.53; 4.50 (reduced, at 550, 520, 410 mμ) and 0.30; 3.60; 0.95; 0.78 (oxidized, at 530, 400, 355 and 280 mμ), compared with 1.00; 0.53; 4.75 and 0.32; 3.0; 1.05; and 0.95 for the purified noncrystalline cytochrome (171). In spite of these similarities the yeast cytochrome differed from that obtained from mammalian sources by several physical and chemical criteria. The spectrum of cytochrome-*c* at liquid air temperatures has been investigated by Estabrook (172). The alpha band shows fine structure with maxima at 549, 546 and 538.5 mμ, while the beta band is similarly split into bands with maxima at 526, 519, and 508.5 mμ. There are indications of three additional bands at 515, 511, and 503 mμ. The Soret band has only one maximum at 414 mμ. Atlas & Farber have presented hydrodynamic data from sedimentation velocity and free diffusion measurements which indicate a nearly spherical structure for the cytochrome from horse, beef, and pig heart (173). Henderson & Rawlinson have determined the E_0 at 25° and pH 6.4 of beef and horse-heart pigment purified by ion exchange chromatography and found it to

be 0.255 v. (174). They have also demonstrated the association of a persistent heme-containing impurity with even the best cytochrome preparations. The tightness of the binding of the impurity onto the cytochrome depends on dialysis conditions, whether alkaline or not. As they point out, this casts doubt on the value of measurements of iron content as the sole criterion of purity.

The isolation of "*c*"-type cytochromes from micro-organisms continues apace. In general these hemoproteins, although containing the same prosthetic group as their mammalian or yeast congeners, differ in several physical, chemical, and enzyme-chemical respects. Thus whether or not they will substitute for the mammalian pigment in typical mammalian assay systems (e.g., cytochrome-*c* reductase, cytochrome-*c* oxidase) is not predictable *a priori*, nor is it to be expected that the mammalian cytochrome will necessarily substitute for the corresponding microbial one with microbial assay systems. This principle is useful to explain such observations as those of Eichel who has studied the DPNH oxidase system in *Tetrahymena pyriformis*, with which mammalian cytochrome-*c* is only slowly reduced and is not oxidized at all. Eichel has therefore concluded that cytochrome-*c* is not a member of the electron transport sequence in this organism (175). Mammalian cytochrome-*c* is certainly excluded, but no such objection would hold against, what would after all be a quite likely conjecture, a *Tetrahymena* cytochrome-"*c*."

Such cytochromes with rather special properties have now been reported in a variety of organisms (1). Recently Lenhoff & Kaplan have isolated a cytochrome of this type from *P. fluorescens* grown at low oxygen tension (176). Its spectrum is similar to the mammalian counterpart but it is not reduced by TPNH in the presence of the mammalian reductase. It is also not adsorbed by Amberlite IRC-50. Its re-oxidation is catalyzed by a cytochrome peroxidase, an enzyme which is inhibited by azide and hydroxylamine (but not CO) at pH 7.0, the pH optimum, but by cyanide only at pH 5.5. The authors suggest that electron transport in this organism, as well as in others devoid of a terminal cytochrome oxidase but containing cytochrome-*c*, may be according to Reaction 17



$$\text{Net: } DPNH + \tfrac{1}{2}O_2 + H^+ \xrightarrow[\text{peroxidase}\ \text{cyt-}c]{\text{flavoprotein}} DPN^+ + H_2O$$

This pathway may hold for the "petite" yeast [Chantrenne (177)] and "poky" *Neurospora* mutants. Cytochromes similar to the one described for *P. fluorescens* have also been isolated by Kamen (178) and by Vernon from *P. denitrificans*, from *M. denitrificans* and from a *Pseudomonad* of unknown species (158, 159). Vernon has shown that these pigments (Λ_{max} reduced at 550, 520, and 416 mμ), like their mammalian counterparts, act as carriers in the cyanide-sensitive oxidation of DPNH and succinate by oxygen. He has also isolated a hemoprotein with the characteristics of cytochrome-c_1, from his nitrate-reducing *Pseudomonad* (159). This cytochrome has Λ_{max} at 553, 523, and 419 mμ and appears to be involved in electron transport from succinate to oxygen.

Two soluble cytochromes of the "*c*" type have been isolated and purified from *Azotobacter vinelandii* [Tissieres & Burris (179)]. Both pigments, called cytochrome c_4 and c_5, are nonautoxidizable, do not combine with CO between pH 4.5 and 13, and are relatively stable to heat and alkali. The Λ_{max} (reduced) for c_4 are as follows: 551 ($\alpha_{mM} = 23.8$), 522 (17.6), 416 (157.2), 315, 270 (20.5); oxidized, 411 mμ (115.8). Cytochrome-c_5 has a very similar spectrum but all peaks are shifted by 4 mμ to longer wavelengths. The E_0' is 0.30 v. for c_4 between pH 5.5 and 7.3, and for c_5 0.32 v. between pH 5.8 and 7.3. The iron content of both cytochromes is 0.46 per cent and their minimal molecular weight approximately 12,000, based on iron content. The authors suggest that the electron transport sequence in this organism is that shown in Reaction 18.

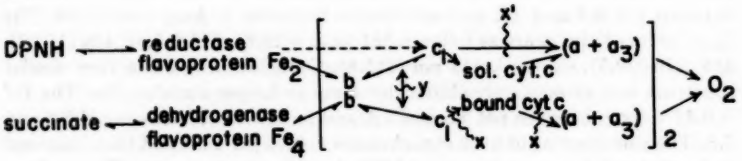
18. DPNH succinate $\rightarrow$ cyt-b_1(Λ_{max} = 560) $\rightarrow$ cyt-c_4 $\rightarrow$ cyt-c_5 $\rightarrow$cyt-a_2(Λ_{max} oxid: 645; red: 630)

Complex, Particulate Electron Transport Systems

Electron transport particles.—Crane, Glenn & Green (180) have prepared from beef heart mitochondria particulate units capable of oxidizing DPNH and succinate at approximately five times the rate of their starting material. DPNH oxidations at maximal rate requires the presence of both phosphate and a metal-chelating agent; phosphate alone is sufficient for succinate oxidation. The preparations contain flavin, cytochromes $a+a_3$, b, c_1, and nonheme Fe and Cu. One type of unit shows the proportions 1(flavin):(total heme Fe):40(nonheme Fe):8(Cu); lipide content 28 per cent. Another type with a lipide content of 67 per cent has molar proportions of 1:8:67:8. In both preparations the ratio of cytochromes $(a+a_3):b:c_1$ is approximately equal to unity. In marked contrast to this constancy of composition the relative content of cytochrome-*c* is quite variable and depends on isolation conditions. Even after fairly extensive removal of the endogenous cytochrome-*c*, no requirement for added cytochrome-*c* can be demonstrated in the case of DPNH oxidation, while succinate oxidation occasionally shows this stimulation. Exposure of the particle to desoxycholate leads to preparations which can readily interact with, and are stimulated by external cytochrome-*c*. This

"opening" phenomenon had previously been described for DPNH oxidase preparations apparently derived from this larger electron transport particle, and showing a similar composition of heme components and associated metal ions [Mackler & Green (181)]. The interactions of DPNH or succinate with O_2, as well as the reduction of externally added cytochrome-*c* are blocked by antimycin A, as is the rapid reduction of all the bound cytochrome components by substrate ordinarily observable with these preparations. Under appropriate conditions the particles may be fragmented to yield preparations similar to the particulate succinic dehydrogenase and cytochrome-*c* requiring DPNH oxidases previously reported by Green *et al.* (181, 182). The picture of electron transport within this particle which emerges from these studies is that it takes place within units as shown in 19, i.e., there is a doublet of two

19.



parallel cytochrome chains capable of shunting electrons at the level of either cytochrome-*b* or -c_1, only one of which still shows an obligatory requirement for (reversibly) bound cytochrome-*c*. The other has been short-circuited by the linkage at x'. Exposure to deoxycholate and other agents irreversibly destroys first the linkages at x and reinterrupts that at x', thus giving rise to the "opening" phenomenon. By treatment of the particle with agents of different severity various transformations may be induced: e.g. the linkage at y may also be broken yielding a cytochrome-*c* requiring DPNH oxidase and a succinic-cytochrome-*c* reductase preparation; or leaving this link intact, that at x' may be severed completely and a combined DPNH and succinic-cytochrome-*c* reductase may be obtained, etc. The nature of the forces binding the various components to the lipoprotein matrix, and the mechanism of electron transfer within the chain remain obscure, as is also the role of the nonheme metal ions.

This scheme, although qualitatively similar, differs in several respects from that proposed by Williams & Chance on the basis of spectroscopic studies on intact respiring mitochondria capable of carrying out oxidative phosphorylation [for reviews see (1) and (183)]. Raw (184) has reconstructed, in part with soluble enzymes, the antimycin-sensitive DPNH-cytochrome-*c* reduction sequence of liver mitochondria and suggests scheme 20, which may be the familiar 20a (184).

20. DPNH → reductase flavoprotein Fe_n → digitonin extract → soluble cytochrome (A_{max}red = 556, 428 mμ) → cytochrome-*c*

20a. DPNH → reductase flavoprotein Fe_n → cyt-*b*- → cyt-c_1 → cyt-*c*

The DPNH-oxidizing system of mammalian skeletal muscle has been isolated in a particulate form and further purified by Lehman & Nason (185). The preparation also shows DPNH- and succinate-cytochrome-*c* reductase activity, is antimycin-sensitive and contains cytochromes-*b*, -*c*, -c_1 and *a* +a_3 and thus appears quite similar to corresponding preparations from other tissues.

Lundegardh (186) has developed sensitive spectrophotometric techniques for the study of respiratory particles *in situ*. In respiring bakers' yeast he has established the main electron-transport sequence as flavoproteins linked to cytochromes of the *b* type, cytochrome-c_1, *c* and cytochrome oxidase in that order. Investigation of the pyruvic oxidase system of *Proteus* by Moyed & O'Kane has disclosed that electrons are passed from a soluble dehydrogenase fraction to a particulate entity containing cytochromes b_1, *a* and a_2 (187). Breummer *et al.* isolated from *A. vinelandii* an electron transport particle similar to the one described for mammalian heart but containing the *Azotobacter* cytochromes enumerated in sequence 18 (188). DPNH-oxidizing systems similar to those described have also been reported by Eichel (175) in *Tetrahymena* and by Humphreys & Conn (189) in lupine mitochondria. Cytochrome-linked oxidation systems for TPNH have been discovered by Jagendorf in chloroplasts (190) and by Tannenbaum in *Acetobacter peroxidans* (191). TPNH is formed by the Hill reaction in the chloroplasts, and by TPN-specific dehydrogenases in Acetobacter. Soluble extracts of germinating seeds have been demonstrated by Mapson & Moustafa (192) to carry out the reactions summarized by 21.

21. $$\text{substrate} \xrightarrow{\text{dehydrogenase}} \text{TPN} \xrightarrow[\text{reductase}]{\text{glutathione}} \text{GSSG} \xrightarrow{\text{dehydroascorbate reductase}}$$

$$\text{dehydroascorbate} \xrightarrow[\text{oxidase}]{\text{ascorbate}} O_2$$

Respiratory inhibitors.—Studies on complex electron transport sequences, as catalyzed by multicomponent particulate preparations, have sometimes been greatly aided by the judicious use of inhibitors. At first these were confined to small iron-porphyrin binding agents such as CN^-, N_3^-, F^-, CO, known to interact with and to block cytochrome oxidase. Later lecithinase A, BAL+O_2, the naphthoquinone SN5949, and antimycin A were used to block the transfer of electrons somewhere between DPNH and succinate on the one hand and cytochrome-*c* on the other. During the past year the latter group of agents has been further enlarged by the inclusion of amytal [Ernster *et al.* (193); Jalling *et al.* (194)], 2-*n*-heptyl-4-hydroxyquinoline-N-oxide [Lightbown & Jackson (195)], and extraction of the particulate preparation with nonpolar solvents, e.g., *iso*-octane [Nason *et al.* (196); Nason & Lehman (197)]. The mode of action of this whole group of inhibitors is still far from clear. Attempts to localize these effects have usually taken the form of either differential activity measurements with various electron acceptors or spectrophotometric examination. The latter technique makes use of the

property, that in the presence of both substrate and acceptor, electron carriers on the reducing site of the inhibitory locus will become completely reduced while those on the oxidizing site will become completely oxidized [Chance (198)]. Although it is tempting to ascribe the mode of action of these agents to binding at a specific site or "factor" in the electron transport sequence, three different modes of interference with the particulate lipoprotein matrix may be operative, either singly or in combination: (*a*) binding onto a component; (*b*) partial or complete removal of a component; (*c*) structural alteration. No rigorous chemical studies on the nature, extent and mechanism of binding have been undertaken to the knowledge of this reviewer, for agents of the second group. Thus the temptation should be resisted and the localization of action limited not to a single component, but, at best, to a specific oxido-reduction involving two components.

Thus antimycin, BAL, and SN5949 all are believed to be involved in blocking electron transfer in the same region, which involves the oxidation of cytochrome-*b* (Fe^{++}) by cytochrome-c_1 (Fe^{+++}) (198, 199). The newly discovered inhibitor 2-*n*-heptyl-4-hydroxyquinoline-N-oxide is a naturally occurring antagonist of streptomycin isolated from *Pseudomonas pyocyanea* and has been synthesized chemically by Cornforth & James (200). It is capable of interfering with the re-oxidation of reduced cytochrome-*b* by the next member of the respiratory chain. In heart muscle and liver mitochondria (where this carrier has been identified by Chance as cytochrome-c_1) it thus resembles antimycin A in its action. The inhibitor is effective also in a wide variety of bacterial species, however, where electron transport is resistant to antimycin action [Lightbown & Jackson (195)]. In a careful investigation of the mode of action of BAL+O_2 and antimycin A on heart muscle succinoxidase preparations Thorn showed that these two inhibitors interact with two different sensitive sites in the preparation rather than on the same site as previously believed (199, 201). This confirms the observation of Green *et al.* (182) that their particulate succinate-cytochrome-*c* reductase was sensitive to antimycin but resistant to BAL+O_2. In the opinion of this reviewer the fact that all the inhibitors of this group are structurally capable of functioning as metal chelating agents and the occurrence of nonheme iron in this region of the electron transport sequence may be more than coincidental.

Nason and his collaborators have adduced evidence that treatment of particulate as well as of bile-salt solubilized DPNH- and succinate-cytochrome reductase preparations with *iso*-octane leads to a lesion in electron transport which can be restored by the addition of certain lipide components (196, 197). The same, or a very similar entity, could be obtained either from the *iso*-octane extract of the particle or from crystalline serum albumin treated in the same way. From the evidence presented it is reasonably certain that this endogenous material capable of releasing the inhibition induced by the earlier treatment is not α-tocopherol. Yet various derivatives of vitamin E restore activity, although a good deal less efficiently on a

weight basis than the lipide obtained from the preparation itself. Some evidence has also been presented that the reaction primarily inhibited is again the re-oxidation of cytochrome-*b* by cytochrome-c_1, i.e., the antimycin sensitive region, and that antimycin inhibition as well could be counteracted by the addition of the lipide components. It would therefore be of great interest to ascertain whether the effects of other blocking agents active in this region and especially those of lecithinase [Nygaard (203)] are similarly counteracted by the addition of the lipide. A possible role of tocopherol in electron transport is also suggested by Bowman & Slater's findings that heart muscle sarcosomes contain 1.1 μM of α-tocopherol (including oxidation products) per gm. compared to $\sim$0.01 μM per gm. for vitamins A, D, and K, and that vitamin E is present even in crystalline bovine serum albumin to the extent of 0.056 μM per gm (204). In liver on the other hand, Green *et al.* have reported a concentration of vitamin K in the mitochondria equivalent on a molal basis to 0.3 that of cytochrome-*c* and 0.2 that of riboflavin (205). Harrison *et al.* have studied the various chemical interconversions of α-tocopherol and its oxidation products, some of which may be of physiological importance (206).

Amytal as well as other barbiturates were shown by Ernster *et al.* (193) and Jalling *et al.* (194) to interfere with electron transport between DPNH (but not succinate) and cytochrome-*c* in liver mitochondria (193, 194). Similar observations on brain mitochondria have previously been reported by Eiler & McEwen (202). Chance believes the amytal effect to be exerted in the re-oxidation of DPNH by the particulate DPNH-cytochrome reductase flavoprotein (198).

Other Hemoproteins

An excellent review of porphyrin and hemoprotein chemistry has been published by Williams (207). The nature of transient intermediates found by George & Irvine in the reaction of hemoglobin, myoglobin, and related hemoproteins with H_2O_2 and other strong oxidizing agents has been discussed previously [(1, 199), also George (208)]. An investigation of some of these compounds by microwave spectroscopy has now been reported by Gibson & Ingram (209). Methemoglobin and metmyoglobin when allowed to react with hydrogen peroxide, potassium periodate, etc., yielded paramagnetic resonance spectra which clearly indicated that the structures formed were those of free radical species derived by hydrogen atom removal from some position of the conjugated porphyrin ring system, and were not caused by a change in valency of the central iron atom (ferryl iron). It has not been established, however, whether the spectra observed were those of compound I (two one-electron oxidation steps removed from methemoglobin) or compound II (one step removed). The resonance observed was similar to that obtained from the oxidation products of metal-free tetraphenyl porphine or phthalocyanine.

Scheler *et al.* (210) have shown in the case of substituted met(ferri)hemo-

globin complexes that there is a positive and continuous correlation between the amount of shift to longer Λ of the Soret band with a similar shift of the adjacent band on the one hand (correlation coefficient $r = 0.884$), and with the amount of "covalent character" as indicated by their paramagnetic susceptibility on the other ($r = 0.924$). Two additional facets are of interest: (a) that as pointed out by Scheler *et al.* the latter values increase in a continuous manner, and do not appear to be grouped only around values corresponding to one ("covalent") and five ("ionic") unpaired electrons [Theorell (211); Hartree (212)]; (*b*) the two functions show the order $F < HCO_2 \simeq CH_3CO_2 < H_2O < OCN < SCN \simeq OH < NO_2 < SeCN < N_3 < CN$ which is approximately the order to be predicted on the basis of their electron-attracting power as measured, for instance, by their substituent constant σ, in the Hammett equation (213).

Oxidative Phosphorylation

The following approaches appear to have been most productive in terms of new findings: the use of refined spectrophotometric techniques, of submitochondrial preparations, of bacterial extracts, and the study of uncoupling agents.

Spectrophotometry.—Two reviews by Chance and his collaborators (183, 198) have appeared recently. The scheme which emerges shows crossover points on addition of ADP to resting mitochondria, identified as sites of initial energy conservation: (*a*) between reduced pyridine nucleotide and cytochrome-reductase flavoprotein, (*b*) between cytochromes-*b* and *c*, and (*c*) between cytochrome-*c* and cytochrome oxidase. These conclusions have now been confirmed by Chance & Williams (214) in the case of sites (*a*) and (*b*) with mitochondria blocked with azide. Some evidence has been presented which interprets part of the ADP concentration necessary to bring the reaction to a half-maximal velocity as a "titration of an endogenous high energy compound in the resting mitochondria." This is conceived as shifting an equilibrium such as 22 to the right in the presence of ADP.

22. $$X \sim I \overset{+Y}{\longleftrightarrow} Y \sim I \overset{P_i}{\longleftrightarrow} Y \sim P \overset{ADP}{\longleftrightarrow} ATP$$

Since the titration value closely approximates the measured concentration of intramitochondrial DPNH the authors identify X in Reaction 22 with DPNH and suggest that in resting mitochondria the latter is present in an inhibited form. This may be identical with the "bound" intramitochondrial form of DPNH reported several years ago to be formed by the oxidation of various substrates in the presence of the tightly-linked intramitochondrial dehydrogenases [Huennekens & Green (215)]. This form does not equilibrate readily with externally added DPNH and is the true substrate of phosphorylating electron transport (*v.i.*). Observations related to these effects have recently been obtained by Ernster *et al.* (193) in their studies of amytal inhibition of DPNH oxidation. Similarly Pullman & Racker (216) have determined that "tightly coupled" mitochondria of mouse liver are incapable of

oxidizing reduced pyridine nucleotides, but will catalyze the active oxidation of such pyridinoprotein substrates as β-hydroxybutyrate and its attendant phosphorylations. The loss of intramitochondrial DPN has been correlated by Hunter *et al.* (217) with swelling and air oxidation of some key site inside the mitochondrion. Bound DPN is inert to the action of *Neurospora* diphosphopyridinonucleotidase [Jacobsen (218)].

Oxidative phosphorylation by mitochondrial fragments.—Particulate, but no longer mitochondrial, systems capable of catalyzing phosphorylation during the oxidation of selected substrates have been obtained by Lehninger *et al.* by digitonin extraction of rat liver mitochondria, and by Green *et al.* by fragmentation of beef heart mitochondria and fractional separation in the presence of ethanol and phosphate (219 to 223). The rat liver preparation catalyzes the oxidation of succinate, added DPNH or β-hydroxybutyrate, by a small particle which contains flavin nucleotides and cytochromes *c*, and $a+a_3$. Only the last named oxidation is accompanied by phosphate uptake into ATP; the maximal P/O was 2.8 with most values lying between 1.5 and 2.4. Phosphorylation also accompanied the partial reactions: β-hydroxybutyrate oxidation by ferricytochrome-*c* ($P/2e^- \simeq 1.0$), and ascorbate oxidation by oxygen in the presence of cytochrome-*c* ($P/O \leq 0.9$). The first step is completely antimycin-sensitive if bound DPN is used as the carrier. On the other hand, reduction of cytochrome-*c* by externally added DPNH is again unaccompanied by phosphorylation and relatively resistant to antimycin action. Similarly the rapid oxidation of added ferrocytochrome-*c* leads to diminished phosphorylation.

ADP is the specific phosphate acceptor for all the reactions; the corresponding derivatives of the other purine and pyrimidine bases are completely inactive. All steps are uncoupled by 2,4-dinitrophenol, pentachlorophenol, gramicidin, arsenate, methylene blue, and dicoumarol. This suggests a role for the last named inhibitor which cannot be related exclusively to its postulated function as an antagonist of vitamin K. As postulated by Martius, vitamin K is supposed to be concerned in the transfer of electrons between DPNH and cytochrome-*b* only (224). Ca^{++} and thyroxine, which have recently been shown to uncouple by virtue of their effect on mitochondrial permeability (*v.i.*), do not interfere with phosphorylation.

The beef heart preparations appear to be largely made up of *cristae mitochondriales* [cf. Palade (225)]. They are capable of catalyzing the oxidation of the various members of the citric acid cycle, of DPNH, and of β-hydroxybutyrate. The oxidation of α-ketoglutarate, pyruvate, malate, and glutamate proceeded preferentially by way of the bound DPN and led to phosphorylation with P/O ratios similar to those obtained with intact mitochondria. Dehydrogenation of isocitrate and β-hydroxybutyrate by added DPN occurred readily but was accompanied by little or no phosphorylation. Succinate oxidation again was completely devoid of phosphate uptake.

Jacobs observed that reduced silicomolybdate or ferrocyanide are suitable electron donors for the study of intramitochondrial phosphorylations

(226). The P/O ratios are of such an order of magnitude (≤ 3 for the former, 2 for the latter) as to suggest that entry into the electron transport chain must occur at the level of bound pyridine nucleotide. The nature of the primary metal complex formed at this site appears to determine not only the rate of electron transfer but the efficiency of energy conservation during this process. It may thus provide a valid model for the processes occurring during the analogous reactions in substrate oxidations.

Phosphorylation in cell-free preparations of microorganisms.—Particulate suspensions of *A. vinelandii* are capable of oxidative phosphorylation. Rose & Ochoa (227) report that the oxidation of H_2, succinate, DPNH, and TPNH by O_2 leads to the transformation of ADP into ATP with very low efficiency ($P/O << 1$). The phosphorylation is essentially unaffected by the concentration of phosphate acceptor and only moderately sensitive to dinitrophenol. Pinchot (228) has further studied the dinitrophenol-insensitive low-efficiency phosphorylating system of *Alcaligenes faecalis*. It consists of a particulate DPNH oxidase, a soluble heat-labile component, and a soluble heat-stable component, tentatively identified as a polynucleotide of relatively low molecular weight. Bovarnick has demonstrated a low level oxidative phosphorylation ($P/O < 0.40$) during the oxidation of glutamate by the Madrid E strain of *Typhus rickettsiae* (229). Among the most useful of the various bacterial systems is one discovered by Brodie & Gray in extracts of *Mycobacterium phlei* and *Corynebacterium creatinovorans* (230, 231). This particulate preparation yields P/O ratios of 1 to 2 with succinate, 1.2 with β-hydroxybutyrate, $\sim$1.8 with pyruvate, malate and fumarate, and 2.5 with α-ketoglutarate, and exhibits the uncoupling phenomenon with dinitrophenol, gramicidin and a variety of other agents previously employed with animal preparations. The particles, which contain cytochromes-*b*, -*c*, and the oxidase, have now been separated from other components of the extract; they may be activated by a partially purified portion of the supernatant fraction. This soluble component has high DPNH-menadione reductase activity [Wosilait *et al.* (232)]. This is of interest in view of the postulated involvement of this enzyme in oxidative phosphorylation [Martius (224, 233)], and the fact that the oxidation of DPNH by menadione (2-methyl-1,4-naphthoquinone) in Brodie & Gray's preparation is inhibited by dinitrophenol and the oxidative phosphorylation by dicoumarol. However, numerous flavoproteins act as menadione reductases; in fact, menadione is frequently an excellent electron acceptor for enzymes of this general class [Mahler *et al.* (234), Boeri & Tosi (160)]. These effects may simply reflect stimulation by some flavoprotein, e.g., DPNH-cytochrome reductase. Thus the reported nonidentity of these supernatant factors with a similar factor reported in *A. vinelandii* by Tissieres & Slater appears quite reasonable (235).

Uncoupling phenomena.—A characteristic feature of the phosphorylating system of mitochondria is its great lability to a variety of agents which have been grouped together as "uncoupling agents," since they permit oxi-

dation to proceed at an undiminished or even increased rate. Several treatments, most notably aging of the particles (especially in the absence of Mg^{++} or Mn^{++}), exposure to Ca^{++} or Zn^{++}, treatment with phosphate, or thyroxine cause primarily morphological alterations in the particles, with diminution or abolition of phosphate uptake as a secondary consequence. The primary event appears to be an increase in the water content of the particles which leads to their swelling and to a profound decrease in the absorbancy of the particle suspension (usually measured spectrophotometrically at 510 mμ). Thus Tapley (236) has been able to establish that Ca^{++} and thyroxine lead to a rapid swelling of the particle while a second group of uncoupling agents, viz. 2,4-dinitrophenol, dicoumarol, and pentachlorophenol even reversed the slight swelling which occurred on brief incubation in the absence of these agents. Since phosphorylation could be completely abolished at appropriate concentrations of the inhibitors, the maintenance of phosphate uptake is not a prerequisite for the prevention of swelling. Brenner-Holzach & Raaflaub (237) have, however, shown that a close relation exists between the intramitochondrial ATP level and the structural integrity of the particles. Some of the experiments of Beyer *et al.*, as discussed below, have led to similar conclusions. The swelling and uncoupling effects of thyroxine on intact mitochondria can be reversed by increasing the Mg^{++} level, according to Tapley & Cooper (238) who also proved that the primary thyroxine effect must be on mitochondrial integrity, by showing that the phosphorylating particles of Cooper & Lehninger are completely resistant to the action of the hormone. The effects of maintaining mitochondria in a Mg-free medium were studied by Beyer *et al.* (239) and by Baltcheffsky, (240). Both groups agree that the changes in both phosphorylative efficiency and absorbancy at 510 mμ are relatively slight in the first 3 to 5 min. but that by 10 min. there is a dramatic decrease in both. Beyer *et al.* demonstrated that Mn^{++} exerted a protective effect, and that the swelling and loss of phosphorylating ability with succinate as substrate was partially reversed by adding Mn^{++}, ATP, and succinate. Using the techniques of Williams & Chance, Baltcheffsky showed that oxidative phosphorylation with hydroxybutyrate remained optimal for a short period and then declined abruptly after several minutes pre-incubation. Concomitant with the inability of ADP to stimulate respiration, there was a similar effect of dinitrophenol and a two- to fourfold stimulation in the endogenous respiration rate. These phenomena were preceded by a rapid fall of the absorbancy at 510 mμ.

Hunter *et al.* have shown that anaerobicity is another useful device for the protection of mitochondria from swelling induced by aging in the presence of phosphate, arsenite, Ca^{++}, and Zn^{++} (217). In contrast to Tapley's finding Dianzani & Scuro (241) claim that a number of redox dyes and also 2,4-dinitrophenol cause swelling and other structural alterations of mitochondria. This apparent inconsistency requires a scrutiny of the experimental conditions employed by the two groups.

Several new uncoupling agents are achieving prominence. Pentachloro-

phenol at low concentrations (2.5×10^{-5} *M*) is a potent uncoupling agent in oxidative phosphorylation, as demonstrated by Weinbach (242) with particulate preparations from the albumen gland of the aquatic snail, *Lymnaea stagnalis*. In the same tissue it was also shown to stimulate the latent adenosinetriphosphatase in a manner quite reminiscent of dinitrophenol. At very low levels ($\sim5\times10^{-6}$ *M*) a stimulating effect on the latent adenosinetriphosphatase of freshly prepared rat liver mitochondria, and of the rate of ATP hydrolysis with soluble preparations was also observed by this investigator. At higher concentrations (5×10^{-4} *M*) the reagent inhibited the adenosinetriphosphatase activity of damaged mitochondria and of soluble preparations (243). Among a large number of steroid hormones tested, progesterone was the only one to elicit a response at low levels ($\sim5\times10^{-6}$ *M*) as an uncoupler of oxidative phosphorylation and an activator of latent adenosinetriphosphatase in liver mitochondria, according to Wade & Jones (244). Similarly triethyl tin sulfate has been reported to be a specific uncoupling agent [Aldridge & Cremer (245)].

Pullman & Racker (216) have observed that upon aging, mouse liver mitochondria release a potent intrinsic uncoupler of oxidative phosphorylation. This effect can be specifically reversed by the addition of serum albumin. The latter component had already been reported as conducive to maintenance of phosphorylating efficiency [Lewis & Slater (246), Sacktor (247); Watanabe & Williams (248)]. The authors point out the possible identity of their inhibitor with mitochrome, a hemoprotein component isolated by Polis & Shmukler (249) and reported by them to function in electron transport and possibly in phosphorylation between DPNH and cytochrome-*c*. More data are essential to evaluate its possible role in these processes. The reversal of the inhibition by albumin makes it necessary to investigate the possible identity of components and effects in the test systems of Pullman & Racker (216), and of Nason *et al.* (197).

Photosynthetic phosphorylation.—Arnon *et al.* (250, 251) define this process as the anaerobic phosphate uptake attendant upon the re-oxidation of the reducing by the oxidizing product formed in the biochemical photolysis of water. It is catalyzed by the electron transport system of intact or broken chloroplast preparations. The postulated pathway is shown in Reactions 23 a and b.

23a. $$H_2O \rightarrow 2[H] + [O]$$

23b. $$2[H] \rightarrow FMN \rightarrow \text{Vitamin K} \rightarrow \text{ascorbate} \rightarrow \text{cytochromes} \rightarrow [O]$$

Ordinary oxidative phosphorylation which accompanies the oxidation of reduced pyridine nucleotides by O_2 also occurs in some preparations but is localized in the mitochondria.

The first demonstration of photosynthetic phosphorylation in bacterial extracts was provided by Frenkel with a preparation from *Rhodospirillum rubrum* (252). Williams reported similar observations with some extracts

of *Chromatium* and of *Chlorobium limicola* (253). As in all other systems of this group phosphate uptake is light-dependent and proceeds under anaerobic conditions.

Free energy of ATP hydrolysis.—Since the conversion of ATP into ADP $+P_i$ is involved in many phosphorylation and energy transfer reactions the estimation of thermodynamic constants for this process is important (Equations 24 and 25).

24. $$ATP^{-} + H_2O \rightleftharpoons ADP^{-} + P_i \quad K_{eq} = [ADP][P_i]/[ATP][H_2O]$$

The standard free energy, ΔF°, for reaction 24 and its enthalpy change ΔH, which can be measured experimentally, are derivable from the K_{eq} by standard thermodynamic manipulations (Equation 25).

25a. $$\Delta F^\circ = -RT \ln K_{eq}$$

25b. $$\Delta H = \partial \Delta F^\circ / T / \partial (1/T) = \Delta F^\circ + T\Delta S$$

In general, the values for ΔF° and ΔH quoted, (with the activity of H_2O taken as unity and those of the other reactants as equal to their total concentrations) have been estimated at 10 to 12 kcal. per mole. This value, relatively high compared with those determined for the hydrolysis of other organic phosphates, has been the basis of the designation of the pyrophosphate bonds in ATP and other mixed anhydride linkages as "high energy bonds" or, more recently, ATP and other molecules in mobile equilibrium with it, as "energy-rich compounds." [Lipmann (254); Meyerhof & Oesper (255); Oesper (256); Hill & Morales (257)]. Podolsky & Morales (258) have now measured ΔH by direct calorimetry for the ATP hydrolysis catalyzed by myosin at pH 8.0 in a wide variety of buffers. At this pH the reaction is that shown in 26, and thus a major contribution of the value of ΔH, as actually measured, is made by the enthalpy of ionization of the various components involved

26. $$ATP^{3-} + H_2O \rightleftharpoons ADP^{2-} + 2H^+ + P_i^{=}$$

(including the buffer). By correcting for these effects they find that ΔH for reaction 24 is -4.7 kcal./mole and they estimate ΔF° for the same reaction at $\sim$6 kcal./mole. This represents a major downward revision for these values, but is consistent with those recently calculated from equilibrium measurements on three different reaction sequences, and with those found for the hydrolysis of a variety of inorganic polyphosphates (See Table II).

Thus a re-evaluation of the magnitude of ΔF° for Reaction 24 is in order, and to this reviewer a value of $\sim$7 kcal./mole appears to be consonant with the facts. This will necessitate a similar revision downward of the ΔF° of hydrolysis of all the other "energy-rich compounds," which have been derived on the basis of ΔF° for reaction 24. Thus, for the guanidinium phosphates and acyl CoA derivatives, $\Delta F^\circ = 8.5$ kcal./mole, while for enol and acyl phosphates $\Delta F^\circ = 11$ kcal./mole.

TABLE II

FREE ENERGY OF ATP HYDROLYSIS
[Adapted from Podolsky & Morales (258)]

Reaction	$-\Delta F°$, 20°C., pH 7* kcal./mole	$-\Delta H$ kcal./mole	Reference
24	9.6	~8.5†	(256)
24	8.3	~7.5†	(259, 260)
24	6.9	~6†	(261, 262)
24	~6.5†	4.7‡	(258)
$P-P_i^4+H_2O \rightarrow 2P_i^{2-}$	~6.5†	<5.8	(263)
$[P-P-P]^{3-}+3H_2O \rightarrow 3P_i^{2-}$	~7†	<6.2§	(264)

* Measured at pH 8.0 (Reaction 26).

† Calculated on the assumption that, empirically, $T\Delta S$ for these reactions ~1 kcal./mole (264).

‡ At 8 pH 8.0 (Reaction 26) all values are raised by 1 kcal.

§ Per pyrophosphate bond split.

THE CITRIC ACID CYCLE

Oxidative enzymes.—This paragraph is limited to reports on pyruvic and α-ketoglutaric dehydrogenase. Juni & Heym have reinvestigated the acyloin condensations catalyzed by pyruvic dehydrogenases from animal tissues and bacteria (265). In the case of pyruvate, their results are interpreted in terms of reactions 27 (*a* to *c*), i.e., that formation of acetoin has an absolute requirement for free acetaldehyde. Addition of acetaldehyde was found to stimulate acetoin formation twelvefold and to inhibit the formation of acetolactate.

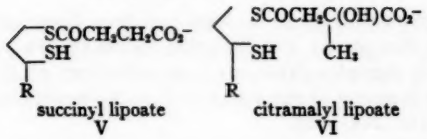
27a. $CH_3COCO_2^- + [Enz \cdot Mg^{++} \cdot DPT] \rightarrow [CH_3CO]^- \cdot [DPT \cdot Mg^{++} \cdot Enz] \xrightarrow[+CO_2]{H^+} CH_3CHO + [Enz \cdot Mg^{++} \cdot DPT]$

27b. $CH_3COCO_2^- + [CH_3CO]^- \cdot [DPT \cdot Mg^{++} \cdot Enz] \xleftarrow{H^+} CH_3C(OH)(COCH_3)CO_2^- + [Enz \cdot Mg^{++} \cdot DPT]$

27c. $CH_3CHO + [CH_3CO]^- \cdot [DPT \cdot Mg^{++} \cdot Enz] \xleftarrow{H^+} CH_3CHOHCOCH_3 + [Enz \cdot Mg^{++} \cdot DPT]$

Pyruvic dehydrogenase is inhibited by 10^{-3} *M* fluoropyruvate [Gal *et al.* (266)]; anaerobically, the formation of acetoin and acetyl CoA are both inhibited by furacin, a nitrofuran derivative [Paul *et al.* (267)].

A novel inhibitor of α-ketoglutaric dehydrogenase activity has been discovered by Montgomery & Webb in a study of the reactions of the cycle in rat heart sarcosomes (268). It is parapyruvate, the dimerization product of pyruvate, and its site of action localized as competitive to succinyl lipoate (V), presumably by the formation of citramalyl lipoate (VI) (269).



succinyl lipoate V citramalyl lipoate VI

Succinic thiokinase.—The enzyme responsible for substrate level phosphorylation in animal tissues catalyzes reaction 28, as demonstrated by Sanadi *et al.* who showed that the formation of ATP from succinyl CoA was a two-step process (270). In this sequence succinic thiokinase functions optimally with the guanosine derivatives, less efficiently with inosine derivatives, while those of adenosine, uridine, and cytosine are inert. Subsequent transfer to the adenosine system (Reaction 29) is catalyzed by nucleoside diphosphate kinase, recently isolated from kidney cortex by Gibson *et al.* (271).

28. $$\text{succinyl CoA} + \text{GDP} + \text{P}_i \rightleftharpoons \text{succinate} + \text{CoASH} + \text{GTP}$$

29. $$\text{GTP} + \text{ADP} \rightleftharpoons \text{GDP} + \text{ATP}$$

Fumarase and aconitase.—Fumarase, is stereospecific in the addition and removal of the hydroxyl group in malate. It is similarly specific with regard to the addition or removal of the proton, as shown by deuterium incorporation experiments in D_2O (Fisher *et al.* 272). This reaction is rapid when compared with the overall reaction, and thus a rapid and stereospecific hydrogen exchange of monodeuteromalate could be postulated, and verified experimentally. The course of the process thus appears to be that of Reaction 30.

30. $$\text{fumarate} + \text{E} \overset{\text{fast}}{\rightleftharpoons} \text{E}\cdot\text{fumarate} \underset{-\text{OH}^-}{\overset{+\text{OH}^-}{\rightleftharpoons}} [\text{E}\cdot\text{intermediate}]^- \underset{-\text{H}^+}{\overset{+\text{H}^+}{\rightleftharpoons}} \text{E}\cdot\text{malate} \overset{\text{fast}}{\rightleftharpoons} \text{E} + \text{malate}$$

Thus a carbanion-type of intermediate is postulated here for an enzyme devoid of a requirement for cofactors. In contrast thereto, the mechanism of the reaction catalyzed by aconitase, an enzyme which requires the presence of ferrous iron and possibly a chelating agent as well, is conceived to proceed via a carbonium type of common intermediate, according to Speyer & Dickman (273). Until recently the activators of aconitase (cysteine, ascorbate, etc.) (see Reaction 31) all combined the action of reducing and chelating

31. $$\text{isocitrate} + \text{E}\cdot\text{Fe}^{++} \rightleftharpoons \text{E}\cdot\text{Fe}^{++}\cdot\text{isocitrate} \rightleftharpoons \text{E}\cdot\text{Fe}^{+}(\text{OH})\cdot\text{intermediate}_1{}^{+} \underset{+\text{H}^+}{\overset{-\text{H}^+}{\rightleftharpoons}}$$

$$\text{E}\cdot\text{Fe}^{+}(\text{OH})\cdot\text{aconitate} \underset{+\text{OH}^+}{\overset{-\text{OH}^-}{\rightleftharpoons}} \text{E}\cdot\text{Fe}^{++} + \text{aconitate}$$

$$\text{citrate} + \text{E}\cdot\text{Fe}^{++} \rightleftharpoons \text{E}\cdot\text{Fe}^{++}\cdot\text{citrate} \rightleftharpoons \text{E}\cdot\text{Fe}^{+}(\text{OH})\cdot\text{intermediate}_2{}^{+}$$

$$\text{intermediate}_1 \left(-\overset{\text{H}}{\overset{|}{\text{C}}}-\overset{+}{\overset{|}{\text{C}}}-\right) \rightleftharpoons \text{intermediate}_2 \left(-\overset{+}{\overset{|}{\text{C}}}-\overset{\text{H}}{\overset{|}{\text{C}}}-\right)$$

agents. These two possible functions have now been dissociated by Her *et al.* who have shown that strong iron-chelating agents (9,10-phenanthroline, 8-hydroxyquinoline, pyrophosphate, etc.) are inhibitory to the enzyme, and that the primary function of the activator is to maintain the iron in the divalent, active state (274, 275).

Occurrence of citric acid cycle.—Research on the enzymes catalyzing the reactions of the cycle, and on the occurrence of these reactions in a variety of organisms and preparations derived therefrom continues actively. As examples may be mentioned the work of Beevers & Walker (276) on mitochondria from Ricinus seeds, of Linnane & Still (277) on mitochondria from yeast, of Godzeski & Stone (278) on a preparation from *Penicillium chrysogenum*, and of Elsden & Ormerod (279) on one from *R. rubrum*. An ingenious method was used by Gilvarg & Davis (280) to demonstrate the cycle in the terminal oxidation of carbohydrate and of acetate in *E. coli* and *Aerobacter aerogenes*. Biochemical mutants which were blocked only in the oxalacetate-acetyl CoA-condensing-enzyme reaction were isolated. They exhibited a nutritional requirement for glutamate or α-ketoglutarate, a virtually complete loss of the ability to metabolize acetate (but not succinate), and a decreased ability to oxidize glucose to CO_2.

Alternate Metabolic Cycles

The Knoop-Thunberg-Wieland cycle.—The key reaction in this postulated cycle, i.e., the "back to back" condensation of acetate to succinate has now been demonstrated in extracts of *T. pyriformis* by Seaman & Naschke (281); see reaction 32.

32. $\text{succinate}^{=} + 2\text{CoASH} + \text{DPNH} + 3\text{H}^{+} \rightleftharpoons 2\ \text{acetyl CoA} + \text{DPN}^{+} + \text{H}_2\text{O}$

The isocitritase cycle.—Two new enzymes have recently been discovered in microorganisms, and their properties determined. One has been called citritase or citrate desmolase and catalyzes reaction 33. It is found in anaerobically grown *Streptococcus faecalis*, *E. coli* and *A. aerogenes*. Its purification and properties have recently been investigated by Dagley & Dawes (282), Wheat & Ajl (283), and Smith *et al.* (284).

33. $\text{citrate}^{3-} \xrightleftharpoons{Mg^{++}} \text{oxalacetate}^{=} + \text{acetate}^{-};\ \Delta F^{\circ} = \sim 200\ \text{cal./mole}$

Of even greater interest is an enzyme recently discovered in aerobic, and facultative bacteria, as well as in fungi [Saz (285); Smith & Gunsalus (286), Olson (287), Campbell *et al.* (288), Wong & Ajl (289)]. The reaction catalyzed was recently investigated by Saz & Hillary (290) and by Smith *et al.* (284). The stoichiometry, equilibrium position, and requirements are indicated in Equation 34.

34. $\text{D-isocitrate}^{3-} \xrightleftharpoons{-SH, Mg^{++}} \text{glyoxylate}^{-} + \text{succinate};\ \Delta F^{\circ} = \sim 2000\ \text{cal./mole}$

It is apparent that this reaction provides a bypath around the isocitric dehydrogenase, the relatively irreversible α-ketoglutaric dehydrogenase, and

the succinic thiokinase reactions of the citric acid cycle. In the presence of appropriate systems capable of metabolizing glyoxylate it might provide alternate pathways for the production, utilization, and oxidation of two-carbon compounds. A scheme such as that shown in Figure 1 might be envisaged.

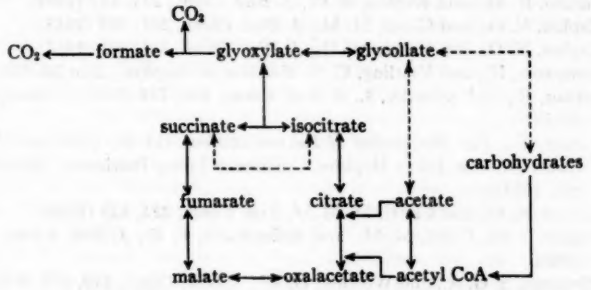


FIG. 1. *Iso*citritase-Dependent Reactions

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NONOXIDATIVE, NONPROTEOLYTIC ENZYMES[1,2]

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The development of the subject matter for this review has provided an exercise in judicious discrimination. Obviously literature which deals with the subject matter covered in other chapters had to be eliminated. To attain a unified approach, all but hydrolases and related transglycosylases were eliminated, together with several fascinating developments in kinetics, phosphate metabolism, decarboxylation, hydration, and isomerization. Further consideration of space made it imperative to eliminate, with regret, sections on the choline esterases and the nucleases; the past year's literature on each of these would constitute individual reviews.

This review discusses what might be considered to be the purely enzymological aspects of a selected group of hydrolytic enzymes not covered in other chapters of this volume, thus eliminating, for the most part, the role of members of this group in physiological, pathological, and histological fields (with exception of reference to their intracellular distribution as revealed by differential ultracentrifugal techniques).

If one were to select a theme that threads through and integrates the ensuing discussion, it would involve the experimental evidence for the multienzyme nature of particular enzyme activities, apparent not only in different organisms, organs, and tissues but also at the intracellular level. Whether this enzymatic microheterogeneity represents a prerequisite function for proper cell metabolism or whether it is a consequence of artifacts arising from maltreatment of the cell by the enzymologist cannot be ascertained from present knowledge of subcellular enzymatic events.

Carboxylic Esterases

Lipoprotein Lipase.—One of the most active developments in the lipase field has been the identity and characterization of the postheparin plasma-clearing factor. Activity in this field stems from the observation of Hahn

[1] The survey of the literature pertaining to this review was completed in November, 1956.

[2] The following abbreviations are used in this chapter: ADP for adenosinediphosphate; AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; DFP for di-isopropyl fluorophosphate; DP for degree of polymerization; DPN for diphosphopyridine nucleotide; FDP for fructose-1,6-diphosphate; G-1-P for glucose-1-phosphate; G-6-P for glucose-6-phosphate; k_1, k_2, k_3 for the velocity constants (respectively) of the formation of enzyme-substrate complex, reversion of the enzyme substrate complex to reactants, and decomposition of the complex to products and free enzyme; K_m for the Michaelis-Menten constant; PP for inorganic pyrophosphate; TPN for triphosphopyridine nucleotide; and V_M for maximum velocity at saturating concentration of substrate.

(1) that injection of heparin into fat-fed animals resulted in the disappearance of lipemia as evidenced by the clearing of the plasma. Recent work has been directed toward (*a*) establishment of this "clearing factor" as an enzyme entity, lipoprotein lipase (*b*) differentiation of this factor from other tissue lipases and esterases, and (*c*) purification. Thus Young & Freeman (2) showed that the injection of heparin into mice resulted in a decrease of glyceride and an increase of free fatty acid in the lymph. Kubie *et al.* (3) developed a convenient technique for demonstrating the appearance of free fatty acid in rat serum by measuring the depression of the second part of the polarographic protein double wave of the albumin. This depression was found to be characteristic of the effect of free fatty acid. By separating heparin from the newly formed lipoprotein lipase with ion exchange resins, Robinson (4) was able to show that heparin appears to be closely bound to the enzyme and that added heparin in amounts as small as 0.1 μg. per ml. could stabilize the labile deheparinized enzyme. On the basis of observations that a bacterial heparinase could partially inactivate lipoprotein lipase, Korn (5) concluded that the latter enzyme is probably a mucoprotein which contains a mucopolysaccharide similar to heparin. Lipoprotein lipase is inhibited by thyroxin [Shore (6)], a surface-active agent [Schotz & Scanu (7)], and by excess heparin, protamine, and several salts [Hollett & Meng (8)]. Pancreatic lipase, which also exhibits a clearing effect [Overbeek & Van der Vies (9)], is not inhibited by these chemicals.

Nikkila & Pesola (10) obtained a four hundredfold purification of lipoprotein lipase by adsorption on calcium phosphate, followed by precipitation at pH 5.5. A similar procedure combined with precipitation by ammonium sulfate resulted in a five hundredfold purification [Hollett & Meng (11)]. In the latter case the apparent development of activity during purification, interpreted by the authors as arising from the elimination of an inhibitor, may be related to the necessarily empirical assay procedure. A lipoprotein lipase which resembles the clearing factor of postheparin plasma has been obtained from heart and adipose tissue [Korn & Quigley (12)]. For full activity, the latter enzyme requires the presence of a protein such as serum albumin and Ca^{++} or NH_4^+. Engelberg (12a) has presented evidence that an endogenous lipoprotein lipase containing heparin is frequently present in human sera prior to heparin injection.

Lipase.—An enlightened approach to the specificity of pancreatic lipase towards glycerides of the higher fatty acids is afforded by the work of Mattson & Beck (13). By actually separating and identifying the products of lipase action on a series of substrates of known structure, they were able to show unequivocally that under their conditions the hydrolysis proceeds stepwise from triglyceride to 1,2-diglyceride to 2-monoglyceride and then ceases. Pancreatic lipase thus seems to be specific for the hydrolysis of glycerol esters of fatty acids esterified at the primary hydroxyl groups. Mattson (14) suggests that this specificity can be employed to investigate the structure of triglycerides. In a very similar investigation Savary & Desnuelle

(15) concluded that the principal pathway of pancreatic lipolysis was identical with that proposed by Mattson & Beck. However, based on the detection in lipase digests of 1-monoglycerides, Savary & Desnuelle concluded that the enzyme did possess a limited specificity for the hydrolysis of the 2-position. They reject the possibility that isomerization had occurred and assume that the hydrolysis is due to the action of but one enzyme.

One may also suspect that bile salts, which were not used by Mattson & Beck, might activate lipase with respect to hydrolysis of the ester bond at the secondary hydroxyl group. This possibility is rendered rather remote by the findings of Wills (16), which strongly suggest that the sole function of bile salts is confined to substrate emulsification and is not involved in an activation mechanism as had been previously suggested. This conclusion is based upon a comparison of bile salt action with that of a series of anionic, cationic, and nonionic detergents. Wills found that only cationic detergents, within a narrow concentration range corresponding to the minimum required for complete emulsification of the fat, were effective in accelerating lipase action. Other conditions or detergents actually inhibited the reaction in most cases.

Others inhibitors of lipase action have been reported: cottonseed extract and hemoglobin inhibit pancreatic lipase [Tauber (17)]; wheat-germ extracts inhibit fungal lipase; sulfhydryl reagents inhibit wheat-germ lipase but not the enzyme activities of aspergillus and penicillium [Dirks *et al.* (18)]. On the other hand various substances have been reported to exert "activating" effects: Carbapol 94, gum acacia, a proteinase inhibitor fraction from soybean [Tauber (17)], gum arabic [Desnuelle *et al.* (19)], and mercuric chloride [Manus & Bendixen (20)].

Further characterization of lipases from a variety of sources have appeared in the literature. Thus Harper (21) found that the so-called lipase from milk acted more readily on butyric acid esters than on true fats in comparison with pancreatic lipase, indicating that the milk-lipase complex may be more closely related to esterases than hitherto suspected. In confirmation of previous observations, Dirks *et al.* (18) found that the heat-inactivation-time curve for wheat-germ lipase as well as for fungal amylase followed second-order kinetics in contrast to the first-order thermal inactivation curves found for most enzymes. In contrast to the relatively heat-labile penicillium enzyme (18), the exolipase of staphylococci was found to resemble such enzymes as myokinase and ribonuclease in its heat-stability properties [Davies (22)].

Mention may be made of two rather new and useful approaches to the assay of lipase. Forster *et al.* (23) found that in contrast to phenol esters of the fatty acids, butyryl, caproyl, and caprylyl esters of 2-naptholsulphonic acids do not yield high blanks in lipase assays due to nonenzymatic hydrolysis. Luchsinger *et al.* (24) describe a very sensitive method for lipase assay based upon the detection and estimation of free glycerol formed from lipase action on mono-olein. As little as 0.2 μg. of glycerol (estimated from the pro-

duction of formaldehyde after periodate treatment) could be detected in the enzyme digest.

Esterases.—Recent progress in esterase enzymology has centered upon the recognition of multienzyme esterase complexes with varying specificity patterns and is especially marked by the utilization of the organophosphorus esterase inhibitors.

Schulte & Engelhardt (24a) have found that an enzyme preparation from liver can hydrolyze the methyl esters of alkyne and alkene 6-carbon fatty acids as well as the fully saturated caproic acid. Using the latter as a standard, they found that unsaturation in the —2,3— and —5,6—positions resulted in a slower rate, whereas unsaturation in the —4,5—position resulted in a faster rate of hydrolysis especially in the *trans*-configuration. The retardation of hydrolysis due to -2,3-unsaturation was found to parallel an increase in the polarity of the double bond group, whereas the decreased rate due to —5,6-unsaturation was ascribed to an unspecified inhibition. In general, introduction of unsaturation results in lower K_m values, which the authors interpret as increases in an "affinity constant." Perhaps a brief discussion on the meaning of the Michaelis-Menten constant in terms of affinity is appropriate at this point. The following is meant to apply in several of the instances to be discussed subsequently.

As pointed out by Hogness & Niemann (25) it is not permissible to identify the reciprocal of the steady-state constant, $K_m=(k_1+k_3)/k_2$, with the equilibrium constant of the reaction between enzyme and substrate (k_1/k_2), as the term "affinity constant" implies. This arises from overwhelming evidence that K_m is a steady-state constant involving k_1, k_2, and k_3 [for a recent excellent discussion of enzyme kinetics see Alberty (26)]. Hence, unless it is proposed to make the term "affinity constant" synonymous with the reciprocal of K_m, discussion of the implication of variations of K_m values lose a certain degree of pertinence.

White (27) found that a partially purified liver esterase preparation was active towards triacetin but was inactive towards other acetate esters. Rat-liver mitochondria have been reported to be a source of an esterase specific for succinyl monocholine quite distinct from aliesterase and acetylcholine esterase, based on a substrate specificity study [Greenway & Quastel (28)].

Oosterbaan *et al.* (29) isolated, by proteolysis, a single radioactive peptide from DFP^{32}-treated liver aliesterase whose amino acid composition pattern closely resembled that of similar peptides which were obtained from α-chymotrypsin and from trypsin. The authors suggest that the DFP-binding site on the enzyme is concerned with the activation of the enzyme substrate complex, whereas another site, whose configuration and amino acid composition varies from enzyme to enzyme, is responsible for binding the substrate and gives rise to specificity differences. In this connection, Bruice & Schmir (30) have shown that imidazole and several of its derivatives can accelerate the hydrolysis of *p*-nitrophenol acetate. From a study of relative rates and the effect of pH they have suggested that the mechanism involves either a

direct attack of imidazole at the carbonyl group of the ester bond or the generation of a hydroxyl group by the removal of water. Further evidence for the participation of the imidazole group in the activity of liver esterase has been presented by Bergmann *et al.* (31), who calculated a pK_a value = 6.2 to 6.5 in the range of that of the imidazole group of the esteratic site as calculated from the pH dependence of the inhibitory effect of tetraethyl ammonium ion.

Further evidence for the existence of two active sites on the molecule of an esterase from liver has been obtained by Kistiakowsky & Mangelsdorf (32a). After correcting for alkali inactivation and working with digests of low ionic strength, the authors found a non-Michaelis-Menten dependence of reaction rate on substrate concentration which could be most easily explained on the basis of two active sites per enzyme molecule. However, the authors suggest an alternative explanation closely related to the theme presented in the introduction to this review, according to which variations and deviations in kinetics may arise from liver impurities associated with the enzyme purification. The resulting microheterogeneous enzyme complex of several molecular species, exhibiting deviation from classical Michaelis-Menten kinetics, would then be difficult to purify and crystallize. Similar explanations may apply to other properties of other enzymes, as will be discussed subsequently.

Myers *et al.* (32) found an inhibitor-insensitive tributyrinase in sera from six species of mammals injected with heparin. The enzyme does not seem to be identical with the postheparin clearing factor but may be related to phosphoorganic inhibitor resistant "A-esterase" [Aldridge (33)]. By zone electrophoresis plasma A- and B-esterases could be separated from each other and from choline esterase [Goutier (34)]. Injection of DFP^{32} resulted in chromatograms of sera in which the radioactivity was very closely associated with the B-esterase.

Byrde & Fielding (35) have discovered what they consider to be an exocellular A-esterase from the fungus *Sclerotina laxa* from its specificity and resistance to inhibition by organophosphorus compounds. Further proof of the identity of the A-esterases might lie in showing that they possess diisopropyl fluorophosphatase activity (see section on the halogenases) as was found by Goutier (34) for the plasma enzyme. Hoshishima (34a) obtained a crystalline preparation with high esterase activity from avian tubercle bacilli. Yields were too small to permit identification of the activity with the crystals.

The pancreas esterase complex seems to differ radically from that of other tissue as studied by Myers *et al.* (36). One of the enzymes, exhibiting activity only towards salicyl esters, may be a proteinase. Another enzyme exhibits a specificity pattern similar to that of the plasma A-esterase but is inhibited by organophosphorus compounds. The pancreas is also a source of cholesterol esterase activity. Direct evidence that this activity is not another attribute of aliesterase or of lipase action was obtained by demonstrating dif-

ferential destruction of these activities by heat and acid, by pH optima, and by demonstrating an absolute requirement for bile-salts for the cholesterol-splitting activity [Swell *et al.* (37)]. Korzenovsky *et al.* (38) found that free cholic acid was the particular component of the bile salt which was required for activity; other bile salts as well as conjugated cholic acid were completely inactive.

Phosphatases

General.—Axelrod (39) has elegantly summarized the available evidence concerning the mechanism of phosphatase action. It is now fairly clear that the basic reaction involves the transfer of a phosphoryl group to an appropriate hydroxyl group, that of water being the classical but not necessarily the most efficient acceptor. This is in contrast to so-called phosphorolysis, where the transfer involves a scission of the C—O bond rather than the O—P bond and therefore involves the phosphate rather than the phosphoryl moiety. Todd (39a) has suggested that the labilization of the phosphate ester linkage in phosphatase action can be brought about by combination of the ester with an essential pair of adjacent groups in the enzyme. This mechanism is analogous to Todd's work on the chemical labilization of a phosphomonoester linkage. A possible clue to the existence and origin of a multiplicity of phosphatases (and presumably other hydrolytic enzymes) is the speculative suggestion of Racker (40) that intracellular phosphatase action may be a manifestion of the degeneration of highly organized electron transfer enzymes such as glyceraldehyde phosphate dehydrogenase. Under certain conditions this enzyme, devoid of dehydrogenase activity, can hydrolyze acetyl phosphate. From a metabolic viewpoint it would be important to determine whether these "degenerated" hydrolytic enzymes are formed during the normal process of metabolism and protein turnover within the cell or arise as artifacts during preparation.

Bamann *et al.* (41) have extended earlier observations on the phosphatatic activity of cerium and lanthanum salts, whose catalytic action resembles in many respects that of the alkaline phosphatases. Thus the pH optima for a variety of substrates lie in the vicinity of pH 9; also, triesters of phosphoric acid are not cleaved by these rare earth salts. These observations constitute the experimental basis for the theory that at least one free phosphoryl hydroxyl is required for combination with rare earth hydroxides to form an intermediate which is then easily hydrolyzable. By measuring V_m and K_m values for various substances the theory was interpreted in detail. Thus the ease of hydrolysis of monoesters as compared with diesters of aliphatic alcohols was interpreted as due to the greater "affinity" of the metals for the monoester. Observations that hydroaromatic esters are not cleaved were interpreted as owing to the extremely low decomposition velocity of the metal-substrate intermediate. A more direct test of this hypothesis would be to test for competitive inhibition of aliphatic ester hydrolysis by hydroaromatic esters.

The phosphatatic action differs in two important aspects from true phosphatases. One is the finding that hydrolysis rates do increase to a maximum and then decrease with increasing substrate concentration [Bamann & Riehl (42)]. This results in varying relative specificities, depending upon substrate metal ratios, since the shape of the pH vs. activity curve differs for each combination of substrate and metal. Secondly, calculations of turnover numbers yield maximum values of about 0.5 mole of substrate hydrolyzed per minute at 37° by one mole of metal ion. This is several orders of magnitude lower than the catalytic efficiency of enzyme action.

In further studies on the effect of rare earth salts on the hydrolysis of thiamine pyrophosphate Bamann & Schuegraf (43) found that the terminal phosphate is readily split by Ce^{++}, whereas hydrolysis of thiamine monophosphate is accelerated more readily by La^{++}. Also hydrolyzable by these rare earths are arginine phosphate, hydroxyamino phosphates, phosphoproteins, and nucleotides (44). Thus the rare earth metals exhibit a wide range of specificity toward most esters of biochemical interest.

Bamann & Riehl (45) have also raised the classical question of the relative specificities of the group specific phosphatases towards phosphomono- and phosphodiesters. They found that crude extracts of alkaline and acid phosphatase of animal origin can hydrolyze both mono- and diesters and that the hydrolysis of monoesters is inhibited by diesters. Presumably the rates of hydrolysis would have been additive if the activities were caused by different enzymes. However, purified mucosal phosphatase exhibits no diesterase activity (see below).

New phosphatase assay procedures have been developed by Monche (46), using 2,4-carboxyazobenzene phosphate as a chromogenic substrate, and by Lissitzky & Bouchilloux (47) who, after incubating the digestion mixture at the origin of a paper chromatogram, follow the hydrolysis by separation and measurement of inorganic orthophosphate after irrigation and development.

Acid phosphatases.—Much of the work in this field has been concerned with the extension, re-examination, and attempts at clarification of previously investigated effects on activity and stability. Jeffree (48) found that the apparent activation of dilute prostatic phosphatase by polyamino compounds is due to a stabilization via protection against denaturation of the enzyme. From observations on the effect of a series of polyamines and their proteolytic digestion products it was concluded that the protective effect is a result of the presence of widely separated amino groups which counteract the phosphatase-destroying action of the negatively charged surface of the glass containers. This inactivation is presumed to be similar to that caused by molecules such as Suramin and sodium dodecyl sulfate which contain widely spaced acidic groups.

Axelrod & Sewell (49) have extended previous observations on the inhibition of this enzyme by *l*-tartaric acid (but not by its enantiomorph) to other α-hydroxy carboxylic acids. Structural requirements for this inhibi-

tion, which appears to be truly competitive, include a carboxyl and an α-hydroxyl plus a carbonyl group in the β position or attached to a carboxyl, hydroxyl, or carboxymethylene.

Delory *et al.* (50) found that the rate of hydrolysis of a number of phosphate esters increased with increasing acidity of the substrate, with a concomitant lowering of the pH optima. Since the rate measurements were made at only one substrate concentration, K_m values cannot be compared. Such a comparison might help to explain one apparent discrepancy; the comparatively rapid hydrolysis of tribromophenol phosphate whose pK was higher than that of most of the compounds which were tested.

By the use of highly purified phosphatase preparations from yeast and from erythrocytes, Tsuboi & Hudson (51) showed that the properties of these enzymes do not permit their classification under Group IV of the isodynamic phosphatases [Roche (52)], which are defined as being activated by Mg^{++}. For instance, the erythrocyte enzyme is not thus activated after sufficient purification. Of course this does not preclude the existence of a true Group IV phosphatase in erythrocytes which was removed during purification. The data do show, however, that the dominant acid phosphatase of erythrocytes does differ in several details, especially those based on kinetic data, from a similar enzyme present in yeast.

An illustration of the multiple nature of the isodynamic phosphatases is afforded by the study of the effect of 28 traditional phosphatase inhibitors (with 16 different substrates) by Roberts (53), who concluded that the acid phosphatase activity of wheat-leaf extracts reflects the action of several distinct enzymes with quite narrow specificities and inhibitory characteristics which do not fit into the classical scheme for the isodynamic phosphatases. Activation of the acid phosphatase of potato and corn-root extracts by β-indole acetic acid has been observed by Turian (54). The ability of prostatic phosphatase to synthesize phosphate esters has been confirmed and extended to the synthesis of the three isomeric cytidylic acids from cytidine and inorganic orthophosphate [Schneider & Loring (55)]. Yields of these isomers were increased in the presence of organic phosphate, indicating that phosphoryl transfer was involved. The prostate enzyme as well as the acid phosphatase of liver has been reported to be distributed between the cytoplasm and the microsomes, very little being associated with the nucleus [Siebert *et al.* (56)]. By the application of gradient centrifugation Thompson & Moss (57) found that the phosphatase, in a bound form, was associated with cell particles intermediate in size between mitochondria and microsomes, thus confirming previous conclusions of de Duve *et al.* (58), who propose the term lysosome to define this intermediate intracellular fraction characterized by the presence of bound hydrolases which can be activated by a variety of physical treatments, i.e., treatment with the anionic detergent X-100 [Wattiaux & de Duve (59)]. Evidence for the existence of three distinct acid phosphatases in liver is presented by Goodlad & Mills (60). The intracellular distribution of these enzymes showed the same pattern.

The past year has been marked by the appearance of at least one paper dealing with a concerted attempt to isolate prostatic phosphatase by the use of several novel or little-used procedures [London *et al.* (61)]. A nonionic detergent is employed to counteract surface denaturation on fuller's earth which was used as a batch adsorbent. At a later stage the preparation was chromatographed on a cation exchange resin, resulting in a preparation representing an eight hundredfold purification over the original gland (protein basis). This preparation was shown by solubility and heat denaturation curves to consist of one-third enzyme protein and could be only partially crystallized. Apparently increasing the purity of the preparation also increased the tendency for the active protein to form inactive and therefore foreign protein, thus decreasing its tendency to crystallize. This represents an exception to the general rule that increased purity increases the tendency toward crystallization.

Alkaline phosphatase.—The complicated problem of the interaction of divalent metals and other factors that influence alkaline phosphatase has been explored by Hofstee (62) and by Hoare & Delory (63). After studying the effects of Zn^{++} and Mg^{++} on the action of duodenal phosphatase at various pH's in the presence of glycine and Versene, Hofstee attributed his results to a spontaneous reversible Mg^{++}-catalyzed association (above pH 6) of an inactive zinc protein with a nondialyzable coenzyme to yield an active enzyme complex. This hypothesis, which in essence elaborates the two-metal theory of Cloetens (64), does not agree with the conclusions of Hoare & Delory, who conducted a related study of the effects of Mg^{++}, Zn^{++}, and CN^{-} on kidney alkaline phosphatase. They concluded that the Cloeten's theory is untenable and prefer the viewpoint of Thoai *et al.* (65) that Zn^{++} can abolish inhibition by cyanide, which can react with an essential disulfide group in the enzyme molecule. Undoubtedly clarification of this problem will result when kinetic studies can be applied to essentially pure enzymes. Such a preparation may now be available as the result of the purification of intestinal mucosal phosphatase by Morton (66).

Contrary to previous reports Morton could find no evidence for the presence of a nonamino acid prosthetic group of low molecular weight or for the presence of carbohydrate in the enzyme preparation, which appeared to be homogeneous by several criteria. His data led him to suggest that Mg^{++} is involved in the formation of an active enzyme-substrate complex much as metals are involved in other enzymes such as the peptidases [Morton (67)]. "Activation" by amino acids was ascribed to their protective action against denaturation. The enzyme cleaved one phosphate group from TPN but not from DPN, thus confirming the ester nature of the third phosphate linkage in TPN [Morton (68)]. Synthesis of the latter via phosphotranferase activity from creatine phosphate to DPN could not be demonstrated. The lack of action on DPN confirms a previous conclusion that the enzyme hydrolyzes only orthophosphate monoesters but not diesters nor pyrophosphates [compare with the results of Bamann & Riehl (45) cited above]. Furthermore the

enzyme is only slightly inhibited if at all by inorganic pyrophosphate, phosphites and phosphonates. DFP at 0.001 *M* inhibited 28 per cent, in comparison with 50 per cent inhibition of impure mucosal phosphatase by monofluorophosphate [Rapp & Sliwinski (69)]. That this relatively mild inhibition is not related to organophosphorus inhibition of the esterases is shown by their demonstration that fluoride ions are not liberated, nor is fluoride ion liberated during the hydrolysis. The alkaline phosphatase of ox serum was found to be inhibited by allicin, the active principle of garlic [Wills (69a)], which apparently can inhibit most sulfhydryl enzymes.

Specific phosphatases.—Recognition of the hexose phosphatases as mediators of carbohydrate balance in mammalian tissue [viz. Shull *et al.* (70)] has given rise to an increased knowledge of their enzymology. Goodlad & Mills (71) confirmed an earlier suggestion that glucose-1-phosphatase is absent from liver. The release of inorganic phosphate from G-1-P is due to its preliminary conversion to G-6-P by phosphoglucomutase which is present in the soluble fraction of the liver homogenates. The G-6-P is then hydrolyzed by an enzyme reported by de Duve *et al.* (58) to be associated with the microsomal-like fraction. Mokrasch & McGilvery (72) describe the preparation of a highly specific liver fructose-1,6-diphosphatase which hydrolyzes only the C-1 phosphate ester linkage. The purified enzyme, with a turnover number of 4000, is activated and its specificity is changed by proteolysis with papain. The physiological significance of this activation in relation to response of the organism to conditions of stress is the subject of a subsequent paper [Mokrasch *et al.* (73)]. Here is another possible mechanism wherein apparent multienzyme systems may arise, in this case in response to physiological factors. That this class of enzymes may be of metabolic significance in other than mammalian tissues may be deduced from the work of Faulkner (74), who has described an enzyme from silkworm blood which, in contrast to that of mammalian tissue, hydrolyzes G-1-P but not G-6-P, and is inactivated by fluoride but is not activated by Mg^{++}. A cell-free preparation of streptomyces can liberate one phosphate from fructose diphosphate apparently via a specific phosphatase action which was found to be the limiting factor in the oxidation of fructose diphosphate by these extracts [Cochrane & Hawley (75)]. N-acetylglucosamine has been found to be a potent inhibitor of the hexose phosphatase activities of *Escherichia coli* extracts [Faulkner & Quastel (75a)].

Experiments with H_2O^{18} have established that, as with other phosphatases, the P—O rather than the C—O bond is cleaved by bull semen 5′-nucleotidase [Koshland & Springhorn (76)]. Data on the exchange between labeled water with either K_2HPO_4 or adenylic acid demonstrated that the enzyme intermediate must be short-lived with respect to decomposition to products. Kaye (77) found that Zn^{++} inhibited the 5′-nucleotidase of cobra venom presumably by competing with Mg^{++}, an activator for active sites on the enzyme. Apparent activation by ATP is most probably due to its chelation with Zn^{++} in the crude preparation. Roberts (78), by the use of paper chro-

matography, has eliminated the possibility that nucleosides may arise from nucleotides via a purine phosphorylase in wheat leaves and concludes that leaves are sources of true nucleotidase activity.

Perlès (79) found that grain phytase is inhibited by excess substrate, presumably because of a reversible combination of the acid groups of phytic acid with basic groups of the enzyme, which causes an association with inactive protein. This is a typical example of inhibition possibly related to the excess negative charge of the substrate, wherein the role of divalent metal ion may be associated with partial neutralization of this charge.

A 2,3-diphosphoglyceric phosphatase, specific for the hydrolysis of the secondary alcohol ester linkage, has been reported to be present in muscle [Rapoport *et al.* (80)] and in erythrocytes [Mányai & Várady (81)]. The enzyme from the latter source is activated by $NaHSO_3$ and $Na_2S_2O_4$ and requires univalent cations for full activity. In contrast to the muscle enzyme, it is not activated by Hg^{++}.

Black & Wright (82) describe a highly specific phosphatase present in yeast, which hydrolyzes phosphoglyceryl methylthiol ester. The authors suggest that the high specificity and "affinity" of the enzyme may be a requisite for the further metabolism of the thiol ester.

Although space does not permit a complete discussion of the phosphodiesterases (e.g., ribonuclease) it may be appropriate at this point to mention the work of Garilhe & Laskowski (83) on the purification of snake venom phosphodiesterase, involving successive application of chromatographic separations on Amberlite exchange resin and on carboxymethyl cellulose. The purified enzyme hydrolyzed the products of ribonuclease and synthetic phosphodiesters but was devoid of nuclease as well as phosphomonoesterase and nucleoside phosphatase activities.

Polyphosphatases

In accordance with the classification scheme of Hoffmann-Ostenhof (84), enzymes capable of cleaving the P-O-P linkages have been grouped together as a class distinct from C-O-P-hydrolyzing phosphatases.

Pyrophosphatases.—Investigations in this field have recently been given added impetus by the elucidation of many activations induced by nucleoside triphosphate which are accompanied by the liberation of inorganic pyrophosphate [Ellis *et al.* (85); Malkin & Denstedt (86)]. The mechanism of metal ion effects is somewhat clearer in the case of the inorganic pyrophosphatase than in the case of phosphomonoesterases. Thus Robbins *et al.* (87) present clear evidence that activation of rat-brain pyrophosphatase by optimal concentrations of Mg^{++} can be interpreted in terms of the formation of an active enzyme-magnesium-pyrophosphate complex which then decomposes into products of the reaction. The inhibitory effect of excess pyrophosphate is ascribed to the formation of inactive enzyme complexes with either pyrophosphate alone or with more than one mole of magnesium pyrophosphate. Although this hypothesis is a reasonable interpretation of

rather complete kinetic data, it may be somewhat premature in view of the recent report on the existence of two distinct inorganic pyrophosphatases in swine-brain tissue, one of which hydrolyzed both PP and ATP [Seal (88)]. The other, specific for only PP, requires both Mg^{++} and glutathione for maximal activity. Activation by Mg^{++} was found to be dependent on the stage of enzyme purification, substrate concentration, buffer, ionic strength, and on pH. In these and other respects this swine-brain pyrophosphatase resembles that from erythrocytes [Malkin & Denstedt (86)] except that the glutathione (or cysteine) requirement appears only after storage of the erythrocytes or if the enzyme is subjected to a preincubation period. Observations on the noncompetitive nature of inhibition by Ca^{++} led the authors (86) to favor the view that magnesium pyrophosphate is the true substrate for the enzyme. In common with other Mg^{++}-activated, fluoride-inhibited enzymes, the inhibition by fluoride was either "quadratic" or "uncompetitive" [see Alberty (26)], depending upon the concentration of Mg^{++}. This was considered to be evidence that magnesium fluorophosphate rather than a Mg^{++}-enzyme complex was involved.

Naganna *et al.* (89) describe a hitherto unreported unstable Mg^{++}-activated alkaline pyrophosphatase whose presence in plant tissues seems to be ubiquitous. Among many of its properties which sharply dsitinguish it from the well-characterized plant acid phosphatases may be mentioned the extremely low immeasurable K_m value resembling, in this respect, similar enzymes from yeast and mammalian tissues.

Bacterial sources frequently provide examples of enzymes with unusual properties. The pyrophosphatase from thermophiles has been extensively investigated by Marsh & Militzer (90). Kinetic data strongly suggest that Mg pyrophosphate is the essential substrate for this enzyme, but in contrast to other pyrophosphatases, it appears that two molecules instead of one are involved in the enzyme-substrate complex. Furthermore the thermophile enzyme exhibits a very broad pH plateau (5.5 to 9.5). Comparison of thermodynamic constants derived from thermal inactivation curves with those from yeast enzyme suggests to the authors that there may be inherent structural differences which render the thermally stable enzyme more stable.

In addition to an alkaline Mg^{++}-activated pyrophosphatase, cell-free extracts of *Streptococcus faecalis* and other bacterial genera contain an acid pyrophosphatase maximally activated by Co^{++} and histidine but not by Mg^{++}; nor is the enzyme inhibited by fluoride [Oginsky & Rumbaugh (91)].

Meta- and polyphosphatases.—Recent work has demonstrated that distinctly different yeast enzymes are responsible for the hydrolysis of straight-chain tri- and polyphosphates on one hand and for the scission of the cyclic structure of the metaphosphates on the other [Kornberg (92); Mattenheimer (93, 94, 95)]. On the basis of studies employing enzyme fractionation and paper chromatography, Mattenheimer presents evidence that the hydrolyses of di-, tri, tetra-, and possibly even higher polyphosphates are each mediated by a different enzyme. He proposes that a classification scheme in which en-

zymes involved in the cleavage of polyphosphate chains with DP≷10 are termed oligophosphatases and are to be contrasted with polyphosphate depolymerases and meta- or cyclo-phosphatases.

Adenosinetriphosphatase.—The relation of this enzyme to muscle contraction will not be reviewed here. An important theoretical contribution to this field is the work of Cohn (96), who has further substantiated the underlying similarity of the mechanism of hydrolysis via phosphatase action by demonstrating with O^{18} that the cleavage of ATP by Mg^{++}-activated adenosinetriphosphatase occurs between the oxygen and the terminal P of ATP. In a novel approach to enzyme kinetics Green & Sharp (97) have quantitated rates of hydrolysis by an avian leukosis virus on the basis of directly counting the number of virus (enzyme) particles with an electron microscope. On the fairly well substantiated presumption that the virus particle is indeed the enzyme, and on the assumption that there is only one active group per particle, an extremely high turnover number of 5,000,000 was calculated. This is of course an extremely high turnover number for an hydrolytic enzyme, thus indicating that there may be more than one active group per virus particle. This enzyme is unusual in other respects in that it requires both Mg^{++} and Ca^{++} for activity and can dephosphorylate ATP and ITP at equal rates under identical conditions. Green (98) reports that ADP inhibits this virus enzyme competitively, whether one derives the appropriate constants from observed rates at varying substrate concentration or whether one calculates these constants from the integrated rate equation of the course of hydrolysis at one substrate concentration. In a continuation of their studies on the heat-stable adenosinetriphosphatase of thermophilic micro-organisms, Marsh & Militzer (99) have found that the action of the enzyme is restricted to the removal of the terminal phosphate from the Mg^{++}-ATP complex.

Several papers have appeared on the adenosinetriphosphatase of homogenates and mitochondria of mammalian tissues. Wade (100) reports that liver mitochondrial adenosinetriphosphatase is activated by progesterone at concentrations as low as $10^{-6}M$. Other steroid hormones did not activate, nor did progesterone have any effect on the hydrolysis of phosphate esters, AMP and ADP. Pentachlorophenol seems to be a specific activator of rat liver mitochondrial adenosinetriphosphatase whose action differs from the activating effect of dinitrophenol in that it can activate the adenosinetriphosphatase of damaged as well as that of intact freshly prepared mitochondria [Weinbach (101)]. Little or no effect of either reserpine or chlorpromazine on the adenosinetriphosphatase of rat brain homogenates could be detected by Century & Horwitt (102). Naidoo & Pratt (103) describe the activation by Ca^{++} and Mg^{++} of two brain adenosinetriphosphatases, one from homogenates of nerve tissue and one from nonnerve tissue. The former resembles in its metal-activation properties the adenosinetriphosphatase from muscle tissue. Homogenates and cell-free extracts of toad embryo contain a dinitrophenol-activated adenosinetriphosphatase [Maruyama (104)].

This activation was found to be diminished by Ca^{++} and azide but not by Mg^{++}.

Nucleoside diphosphatase.—In contrast to the polyphosphatases hitherto described, an adenosinediphosphatase from potatoes was found to be fully activated at Ca^{++} concentrations greater than 10^{-4}, thus indicating that Ca^{++} forms a complex with the enzyme directly rather than with the substrate [Raaflaub (105)]. At low concentration of Ca^{++}, ADP apparently competes with the enzyme for the available Ca^{++}. Thus the mechanism of the metal ion effect in this case seems to be more closely related to that of the phosphomonesterases than to that of most of the polyphosphatases. Plaut (106) has purified a highly potent Mg^{++}-requiring enzyme from aqueous extracts of rat-liver mitochondria acetone powder, capable of liberating one phosphate from the diphosphates of inosine and guanosine, but not from ADP nor cytidine diphosphate. In contrast to mitochondrial adenosinetriphosphatase, its activity *in situ* is not enhanced by dinitrophenol.

Nucleotide pyrophosphatase.—Apparently an enzyme of this class is responsible for part of the loss of coenzyme nucleotides in freshly prepared liver homogenates [Frunder and Richter (107)] and in mammary gland preparation [Gander *et al.* (108)] but does not play such a role in erythrocytes [Hofmann (109)]. In an interesting discussion of the significance of naturally occurring enzyme inhibitors, Swartz *et al.* (110) traced the apparent thermal activation of the nucleotide pyrophosphatase from *Proteus vulgaris* to the presence of a thermolabile polypeptide inhibitor. The enzyme itself can be protected against heat inactivation by the presence of pyrophosphate or by an unidentified organic phosphate present in the bacterial extracts.

Halogenases

The enzymic dehalogenation of compounds in which the halogen is combined directly to carbon seems in at least several instances to proceed via oxidative, nonhydrolytic mechanisms (111, 112, 113).

In the field of the phosphorus dehalogenases, the past year has been notable by the continuing investigations of Mounter and co-workers (114 to 122). In 1954 Mounter & Dien (114) demonstrated that a hog-kidney enzyme preparation liberated one mole of hydrogen fluoride from DFP. Enzymes with similar specificities seem to be universally distributed in mammalian tissues (115) and in micro-organisms (116, 117). Mounter & Chanutin (118) studied the activation caused by certain organo-nitrogen compounds such as histidine and pyridine derivatives on the one hand and by Mn^{++} on the other. Observations on the cofactors-vs-activity curves led them to presume that these two types of cofactors combine with the enzyme to form an active complex in equimolar proportions. Inhibition in the presence of excess nitrogen-containing cofactor was interpreted as a result of the formation of a complex which contained two moles of cofactor for each mole of Mn^{++},

thus competing with the formation of active enzyme complex. In contrast to the Mg^{++} activation of the phosphatases, combination with substrate does not seem to be involved here, since no inhibition was observed at substrate concentrations where limiting velocities obtained. Further investigation revealed that the activity from many tissues could be resolved into water-soluble and water-insoluble enzymes [Mounter (119)]. An analysis of the pH-vs.-activity curve revealed an ionizing group within the enzyme ($pK = 7.2$), identical with that of acetylcholine esterase [Mounter (120)], indicating that at least one active center is common to both enzymes. Further specificity studies with a variety of phosphonates led Mounter & Dien (121) to propose that the type of compound which is hydrolyzed is determined by the presence of alkyl groups, the relative reactivity of P—F, P—CN and P—O—P bonds, and the geometry of the molecule.

Inasmuch as phosphonates are not normal tissue constitutents, these halogenases, in view of their ubiquity, must have some function other than their action on phosphonates. Mounter (122) now claims from kinetic studies involving pH, inhibitors, mixed substrates, and attempted enzyme separation by paper electrophoresis, that di-isopropyl fluorophosphatase is identical with acylase, capable of hydrolyzing the N-acetyl derivatives of a variety of amino acids. Here, indeed, is an illuminating illustration of the ambiguities of an enzyme classification scheme based upon bond specificities.

Mention should be made at this point of the apparent identity of serum A-esterase with di-isopropyl fluorophosphatase [Goutier (34)]. Hoskin (123) found that the sole hydrolysis product resulting from the action of serum on Sarin (isopropyl methyl phosphoryl fluoridate) is isomethyl phosphonic acid.

Sulfatases

Aryl sulfatases.—Dodgson *et al.* (124) have pointed out that the sulfatases are so widely distributed that they may well possess some important general function beyond being involved in detoxication mechanisms. As with other hydrolases, a multiplicity of enzymes seems to be associated with a particular tissue. Thus three enzymes with aryl sulfatase activity have been separated from human liver tissue [Dodgson *et al.* (124)]. Two of these are soluble and the third is similar in properties to that reported to be present in the microsomal fraction of rat-liver cells [Dodgson *et al.* (125)].

In kinetic studies involving a series of substituted aryl sulfates as substrates for alcaligenes sulfatase Dodgson *et al.* (126, 126a) conclude that the withdrawal of electrons by substituents from the ester sulfate side chain may be an important prerequisite for aryl sulfatase activity. Thus it was found that introduction of electrophilic substituents in the benzene ring of aryl sulfates enhanced K_m and V_M values, whereas these values were decreased by the introduction of nucleophilic substituents. It may be pointed out that these effects are not necessarily related to the "affinity" of the enzyme for the substrate but may reflect the influence of these substituents on the decomposition velocity of the enzyme-substrate complex. The mode of

action of this microbial enzyme seems to be similar to that present in Taka-diastase as revealed by the work of Boyland *et al.* (127), who found that the enzyme could hydrolyze all of a number of aryl sulfates (at one substrate concentration) except those with a basic group in the ortho position. Of the eight esters found to be resistant, at least five are derivatives of carcinogenic amines. They suggest that variations in susceptibility may be related to the ability of these substrates to form *zwitterions*. Dodgson *et al.* feel that such an explanation is not adequate, at least as applied to the alcaligenes enzyme. Dodgson *et al.* (128) have shown that inhibition of the alcaligenes enzyme by sodium cyanide and by hydrazine is anticompetitive; i.e., Lineweaver-Burk plots yield a family of parallel curves with varying inhibitor concentrations. This type of inhibition, which has been interpreted as a combination of inhibitor with enzyme-substrate complex but not with free enzyme, is in accord with the further finding that combination of substrate with enzyme yields a new group ($pK = 7.9$) not present in the free enzyme. Presumably it is this new group which combines with the above-mentioned carbonyl reagents.

The anomalous kinetics exhibited by mammalian sulfatase towards nitrocatechol sulfate, characterized by the absence of zero-order kinetics at saturating levels of substrate, was shown by Dodgon & Spencer (129) to be due to a peculiar feature of the enzyme and not, as had previously been suggested, to polymeric forms of the enzyme nor to the presence of an impurity in the substrate [Roy (130)].

Other sulfatases.—The steroid sulfatase of the mollusc *Patella vulgata*, a rich source of sulfatases of varying specificities, has been highly purified by Roy (131) and shown to act only on 3-β-sulfates of the 5-α- and Δ-5-steroids. Evidence is presented that the hydrolysis of cortisone-1-sulfate is mediated via a glycosulfatase rather than an alcohol sulfatase. Stitch *et al.* (132) also describe the purification of a sulfatase from patella which can hydrolyze *epi*androsterone sulfate. On the other hand, Gibian & Bratfisch (132a) have isolated a highly specific mammalian liver steroidal sulfatase, devoid of β-glucuronidase activity, capable of hydrolyzing the sulfate esters of estrone and dehydro*epi*androsterone, but devoid of activity towards the sulfate esters of androsterone, phenolphthalein, and chondroitin. Degradation of heparin by heparin-adapted bacteria may involve the action of both an alcohol sulfatase and an amino sulfatase according to Korn & Payza (133).

Correcting an error that has persisted in the literature for several decades, Ettlinger & Lundeen (134) have shown by organic chemical degradation procedures that the structure of sinigrin, the prototype of the mustard oil glycosides, is an analogue of a hydroxamic acid derivative rather than a substituted isothiocyanate as depicted in many textbooks. This latter assumption is based on the fact that the hydrolysis product derived from myrosinase, a mixture of thioglucosidase and myrosulfatase, has been shown to be an isothiocyanate. The consequences from this new finding are that myrosulfatase must cleave an N—O—S rather than a C—O—S linkage and that, fur-

thermore, myrosinase must somehow catalyze a modified Lossen rearrangement whereby a derivative of a hydroxamic acid is rearranged to isocyanate, analogous to the well-known Hoffman rearrangement of amides. Whether this rearrangement requires either or both of the enzyme components of myrosinase, or whether it is a consequence of these activities or is an independently enzymically catalyzed reaction remains undecided.

Oligoglycosylases

General.—The present discussion will be limited to those studies in which the glycosidase action is manifested by hydrolysis. Transglycosylation to other than water will be treated in a separate section. Carbohydrase activity patterns have been described in the blow fly larva [Evans & Marsden (135)], in cytoplasmic particles of corn scutellum [Hageman & Hanson (136)] and in marine algae [Duncan *et al.* (137)]. Conchie & Levvy (138) report that a series of specific glycosidases are inhibited by the aldolactones related to the parent sugar of the substrates.

Alpha-glucosidases.—Further studies on mucosal amylo-1,6-glucosidase reveal this enzyme to be a sulfhydryl enzyme with a pH optimum between 7.2 and 7.6 and an ionizable group of $pK = 7.5$, and to be inhibited by debranched phosphorylase limit dextrin [Larner & Schliselfeld (139)]. This enzyme can cleave glucose from the isomaltose linkages present in dextran, glycogen, and muscle phosphorylase limit dextrin (with the liberation of glucose) but is inactive towards isomaltose, panose, and α-amylase limit dextrins. Cori (140) has applied amylo-1,6-glucosidase in conjunction with other enzymes acting on starch to determine the structure and molecular weight of starches and glycogens. A related enzyme, oligo-1,6-glucosidase, capable of hydrolyzing the isomaltose linkage in isomaltose and isomaltotriose, when coupled to a kinase-*Zwischenferment*-TPN system [Larner, (141)], but with no demonstrable action towards amylo-1,6-glucosidase substrates, has recently been isolated from mucosa by Larner & McNickle (142). Wetter (143) has shown that isomaltase and maltase activity of *Aspergillus niger* could be separated from the "limit" dextrinase (also an α-1,6-glucosidase) by paper electrophoresis.

Manners & Maung (144) prepared a yeast enzyme similar to amylo-1,6-glucosidase and to the R-enzyme of potatoes but showing a much broader specificity spectrum, since it was found to attack isomaltose, maltotriose, and panose as well as glycogen and amylopectin. Larner & Gillespie (145) have found that extracts of intestinal mucosa also contain an enzyme, apparently different from oligo-1,6-glucosidase, isomaltase, or maltase, capable of hydrolyzing the α-1,3-linked disaccharide nigerose, recently isolated from acid hydrolyzates of waxy maize amylopectin by Wolfrom & Thompson (146).

Beta-glucosidases.—The β-glucosides of salicylic acid [Hofstee (147)] and 4-methyl umbelliferone [Robinson (148)] have been employed as chromogenic substrates for β-glucosidase assay. Hofstee (147) found that the pH

optimum of almond emulsin depends on the varying influence of K_m and V_M. Variation in the latter was attributed to ionization of a group in the enzyme. It should be apparent by now that one outstanding feature of the past year is the popular trend towards interpretation of pH optima in terms of an ionizable group within the enzyme [Alberty (26)]. Surprisingly, inhibition by cellobiose or by salicin was not influenced by change of substrate concentration. Huang's observation (149) that certain anthocyanins can be hydrolyzed by a fungal enzyme preparation but not by emulsin illustrates the varying specificities of even group specific β-glucosidases from different sources. The kinetics of this hydrolysis as studied by Huang (150) were interpreted to involve enzymic hydrolysis to anthocyanidin and sugar followed by a spontaneous transformation of the aglucon pigment into a colorless form. A cucumber β-glucosidase (elaterase) can cleave but one molecule of glucose from each of several cardiac glycosides, according to Enslin & Rehm (151). According to Manners (152) barley contains a group-specific β-glucosidase in addition to several other β-glucosidases of restricted specificity. In a series of papers Jermyn (153) describes the production, purification, and properties of a β-glucosidase from *Stachybotrys atra*. An interesting feature of the purification procedure involves adsorption on $MgCO_3$ columns. The enzyme, which was found to be quite typical of fungal β-glucosidases with respect to pH optimum, K_m values, and thermal stability, appears to be a group specific β-glucosidase; all configurational alterations of the O-glucopyranose ring led to compounds incapable of being split. In a thorough study of the effect of inhibitors on the enzyme, Jermyn (153) finds typical competitive (i.e., glucose) and "uncompetitive" inhibition (i.e., turanose). He interprets his data on the basis of two binding centers in the enzyme. An interesting application of the theory of Koshland & Stein (154) revealed that the prediction from this theory on the basis of the specificity alterations brought about by varying constituents was valid in general but could not be applied in detail. In several instances the preponderant effect seemed to be related to the rate of attachment of substrate to enzyme. Although Jermyn equates reciprocal K_m values with "affinity" he correctly interprets the finding that the K_m values for the hydrolysis of *p*-nitrophenyl-β-glucoside are not identical with constants obtained when this compound is used as an inhibitor as demonstrating that K_m is not a true dissociation constant.

Invertase.—Most of the recent literature on the invertases relates to their transglycosylative capacity and will be discussed later. After examining the appearance of the reducing sugars which arise from the combined action of yeast and fungal enzymes on sucrose and raffinose, de Grandchamp-Chaudun (155) concluded that nonreversible enzyme-substrate complexes are formed instantaneously, a surmise which is in accord with accumulating evidence in many instances that k_2 is negligible as compared with k_1. The polysaccharide which accompanies yeast invertase during purification has been shown to be identical with the mannan, yeast gum [Cifonelli & Smith (156)].

Beta Galactosidase.—Hofsten (157) describes the purification of a heat-stable enzyme from the ascomycetes *Ophiostoma multiannualatum*, employing ion exchange and zone electrophoresis. Zone electrophoresis can separate several β-galactosidases present in *E. coli* [Aladjem *et al.* (158)]. One of these enzymes can hydrolyze lactose but not *o*-phenyl galactoside.

Glucuronidases.—The physiological role of mammalian β-glucuronidase is exemplified by the recent findings of Fishman *et al.* (159) that injection of crude pituitary extracts into mice raised the level of renal β-glucuronidase. The intracellular distribution of this enzyme activity in rat liver apparently differs from that of other hydrolases in that it was found to be more closely associated with the microsomes than with any other fraction [de Duve *et al.* (58)]. Further analyses of enzyme distribution patterns in conjunction with properties of the enzymic activities indicated that there may be more than one intracellular enzyme although de Duve *et al.* do not rule out the possibility of variable concentrations of activators (i.e., nucleic acids) creating new specificities. Here again is an illustration of the problem of the biological reality and origin of an apparent multienzyme system of restricted specificity.

The mollusc *P. vulgata* is apparently a rich source of β-glucuronidase. Extracts display an activity about 20 times that of most preparations made from mammalian tissues [Stitch *et al.* (132)]. Conchie *et al.* (160) have reported the presence of α- as well as β-glucuronidase activity in this same organism. Evidence that these activities are due to the presence of distinct enzymes is based upon the inhibition of the β- but not the α-enzyme by saccharo-1,4-lactone and upon the relative acid stability of the α-glucuronidase activity. Doyle *et al.* (161) have described procedures for the production and purification of β-glucuronidase from *E. coli*. The enzyme was found to be quite labile in the absence of amino acid chelating agents, cysteine, or a nonprotein fraction of culture fluid supernate.

Mead *et al.* (162) have utilized the fluorescence of free 4-methylumbelliferone for the assay of β-glucuronidase, using nonflourescent β-glucuronide as substrate, an improved synthesis which has been described by Marsh & Levvy (162a). Fishman & Green (163) have developed a very sensitive assay procedure for β-glucuronidase, based upon the reaction of acid napthoresorcinol with conjugated glucuronic acid after the free acid is removed by hypoiodite oxidation to saccharic acid.

Polyglycosylases

Amylases.—Several investigations have contributed substantially to the elucidation of the mechanism of amylase action. The smallest dextrin isolated from a salivary α-amylase digest of amylopectin was found to be a pentasaccharide, in which an α-maltosyl is joined to the reducing end of a maltotriose unit via an a-1,6-glucosidic linkage [Whelan & Bines (164)]. A study of the oligoglucosides formed in the early stage of salivary amylase hydrolysis of members of the malto-dextrin series from the pentaose to the heptaose,

each labeled in the C-1 position at the reducing end of the molecule confirmed the generally accepted view that the hydrolysis is not entirely random [Pazur & Budovich (165)]. The most easily hydrolyzable linkage was that penultimate to the reducing end of these substrates, whereas linkages adjacent to the terminal positions were completely unamenable to attack. Application of this tracer approach should prove quite valuable in the general field of enzymic depolymerizations. By means of viscosimetric measurements Lohmar *et al.* (166) were able to preselect dextrins with reproducible properties by controlled degradation of waxy corn starch with malt α-amylase.

Neufeld & Hassid (167) have confirmed previous observations of Peat *et al.* (168) that β-amylolysis of potato amylose proceeds to approximately 70 per cent unless Z-Enzyme is added. They showed, however, that the Z-Enzyme is identical with neither the group-specific β-glucosidase of emulsin nor with laminarinase (α-1,3-polyglucosidase) and that furthermore the Z-Enzyme effect can be simulated by treatment of the 70 per cent limit amylose β-dextrin with alkali. They conclude that β-amylolysis of amylose proceeds via a mechanism involving both partial and complete removal of amylose chains, resulting in exposure of previously protected alkali-labile groups. It is now clear from a study of ratio of reaction of sweet potato β-amylase on amylopectins and on model singly branched substrates consisting of two homologous series derived from isomaltose and from panose, respectively, that the rate of β-amylase action slows down as the enzyme approaches the branch points, so that isolation of an absolute β-limit dextrin is impractical if not impossible [Summer & French (169)]. Furthermore it was shown that the resistance of a given chain to β-amylolysis is highly dependent on the number of residues present in the branches; thus addition of glucose to a branch rendered resistant a previously nonresistant adjacent chain.

According to Van Dyk *et al.* (170) previously reported dependence of initial velocity of starch hydrolysis by α-amylase at certain substrate concentrations is an artifact arising from a neglect of the dependence of the velocity on the extent of reaction. To circumvent this difficulty they define initial velocity in terms of the reaction time for the hydrolysis of a small fraction of bonds. Application of this approach to the data of the present author revealed no differences in reaction rates when compared to the values obtained by the conventional procedure of estimating initial rates of hydrolysis of amylose by malt α-amylase [Schwimmer (171)]. It may be that measurements were made at early enough stage of digestion to obviate the above-mentioned difficulty.[3] In a subsequent paper Van Dyke & Caldwell (172) determined "affinity" constants ($=1/K_m$) and rate constants as a function of temperature, with waxy maize starch as substrate. Thermodynamic

[3] In apposition to this discussion may be mentioned a recently published procedure for determination of initial rates, objective albeit empirical, in which a statistical approach is applied to results of the fitting of orthogenal polynomials to the data of a particular experiment [Booman, K. A., and Niemann, C., *J. Am. Chem. Soc.*, **78**, 3642 (1956)].

constants comparable with those of other carbohydrases were obtained. Thus activation energies of about 13,000 calories/degree/mole agree well with comparable values for the amylase of various body fluids [Kovacs & Tuba (173)]. On the other hand, according to McGeachin (174), the amylases of various body fluids do differ in their apparent activation requirements for amino acids and protein. The conclusion of the de Duve school that many intracellular hydrolases are associated with intermediate microsome-like particles is strengthened by the finding of Laird (175) that a microsomal-like fraction of pancreas carries most of the amylase activity of this organ. Although this review does not encompass the synthesis of enzymes, in view of a previous controversy concerning the possible role of inositol as a constituent of α-amylase, it may be of interest to mention the finding of Ramachandran *et al.* (175a) that incubation of pancreatic slices with ATP and inositol resulted in increased amylase activity.

Markovitz *et al.* (176) have isolated an electrophoretically homogeneous crystalline α-amylase from *Pseudomonas saccharophilia* by procedures involving adsorption and elution from starch columns, first used successfully for α-amylase purification by Schwimmer & Balls (177). In a discussion of the comparative biochemistry of the purified amylases the authors make the observation that procedures by which an enzyme is purified may affect the properties being compared, so that such intercomparisons must be treated with caution. Here again is an illustration of artifacts that arise and are interpreted in terms of a multienzyme system. However, the appearance of an apparent inflection point at about 15° in the plots used to calculate activation energies does seem to be characteristic of the α-amylases from many sources, including that from *P. saccharophilia*, pancreas, *B. subtilis*, and malt. Recent analyses of such plots of many enzymes that exhibit this type of break have led to the conclusion on both experimental and theoretical grounds that there is actually a gradual change in slope rather than a sharp break [Maier *et al.* (178); Hultin (179)].

Jagger & Hutchinson (180) interpret data on the dry-state inactivation of bacterial amylase by fast-charged particles as an instance of a triple ionization requirement for inactivation. Akabori *et al.* (181) found that radioactivity is apparently incorporated into the molecules of a fungal amylase during the hydrolysis of radioactive starch. It is not clear from the data whether or not this incorporation is an artifact or is related to the mechanism of action of the enzyme.

A classical problem in the amylase field, which may be amenable to solution by modern tracer methods, is that of the difference in the mechanism of action of the α- and β-amylases. The fact that the former produce α-mutarotating dextrins, whereas the latter produces β-mutarotating maltose, has been interpreted by the present author [Schwimmer (182)] in terms of which side of the oxygen of the α-1,4-glucosidic linkage is split. The time is surely now ripe for the application of O^{18} tracer studies in the elucidation of this mechanism.

Cellulases.—Jermyn (153) has summarized evidence for the presence of three distinct cellulolytic enzymes in *Stachbotrys atra*, each acting on increasingly depolymerized products: a "swelling factor" responsible for the opening up of the crystalline structure of the native cellulose, an enzyme which attacks soluble cellulose derivatives and cellodextrins down to cellotriose, and a cellobiase. Whitaker (183), finding that the number-average DP of a cellodextrin decreased but slightly during hydrolysis by a cellulase from the fungus *Myrothrecium verrucaria*, concluded that at least a partially random cleavage of the glucosidic bonds occurred. That this cleavage may not be entirely random follows from the result of a study of the action of this cellulase on labeled cellotriose [Whitaker & Merler (184)]. It was found that the linkage adjacent to the nonreducing end of the cellotriose was hydrolyzed five times faster than that adjacent to the reducing end.

In order to explain the appearance of glucose during early stages of hydrolysis of a barley poly β-glucoside by an enzyme from the same source, Bass & Meredith (185) postulate the presence in barley of both "endo" and "exo" enzymes.

In a rare application of classical collision theory to enzyme kinetics Whitaker (186) concluded that the increase in second-order rate constants (which presumably reflects the formation of enzyme substrate complex) with increasing DP of substrate could best be interpreted as an increase in the steric factor rather than a change in collision number, since the energies of activation were invariant with respect to these substrates. It may be pointed out that the application of second-order collision theory is valid under the conditions of second-order reaction kinetics if it can be shown that k_2 is negligible as compared with k_1, an assumption which seems quite reasonable, as mentioned previously.

The same authors also found that heat treatment of the enzyme resulted in varying activity losses toward the four substrates, which they interpret as owing to alterations in the properties of the enzymes brought about by partial heat denaturation and not to the presence of more than one enzyme, which would be the conventional explanation of this type of data. This interpretation appears to be in conflict with the studies of Miller & Blum (187) and of Hash & King (188), who independently interpreted the zone electrophoresis of crude preparations from the same organism as demonstrating the presence of a multienzyme system. Pertinent to this question is the demonstration by Basu & Pal (189) that previously recommended shaking of cellulase digestion mixtures not only is unnecessary but can indeed lead to discordant results. If the concept of Whitaker that partial alterations of enzymic properties can be brought about by a partial denaturation of the enzyme molecule without destroying its activity is valid, then the whole concept of the multiple nature of hydrolytic enzymes developed in the multitudinous papers discussed in this report may require extensive re-examination.

Concentration and partial purification of cellulases have been reported

from the mollusc *Teredo* [Greenfield (190)] and a wood-rotting basidiomycete *Polyporus palustris* [Higa *et al.* (191)].

Hyaluronidase.—As a result of a comprehensive investigation of the viscosity changes associated with hyaluronidase action, Andersen & Graae (192, 193) have formulated an empirical kinetic equation relating enzyme, time, and degree of reaction, which agrees with an equation derived from the postulation of a three-step reaction. The authors correctly reject the interpretation that this is a manifestation of the combination of three molecules of hyaluronic acid with enzyme. They feel that an explanation based upon a certain degree of nonrandomness of splitting of linkage with respect to position of the linkage and transglycosylation may be involved. This interpretation certainly is in agreement with findings for the hydrolytic cleavage of other polysaccharides.

Evidence that the mechanism of animal hyaluronidase action is quite different from that of bacterial hyaluronidase is afforded by the finding of Schütte & Greilung (194) that the oligosaccharides formed from bacterial hyaluronidase, although apparently containing equal amounts of N-acetyl glucosamine and glucuronic acid, were not identical with the corresponding end products of animal hyaluronidase action. The nature of this difference is now elucidated as a result of the work by Linker *et al.* (195) who showed that hyaluronidase from several bacterial sources split the glycosidic linkage by an elimination reaction, resulting in the formation of -4,5- unsaturation in the uronic acid moiety concomitant with hydrolysis. Apparently transglycosylation is not involved. This type of completely novel reaction may well be involved in other carbohydrate isomerizations. The production and purification of hyaluronidase from bacterial sources with classical fractionation schemes have been reported by several investigators [MacLennan (196), Rogers & Spensley (197), Emmart & Cole (198), and Baker *et al.* (199)].

The finding of Fuson & Marcus (200) that the apparent hyaluronidase activity of bull testes decreased with increasing nitrogen content of substrates isolated from various sources can perhaps be explained as a variation in true substrate concentration due to the presence of nitrogen-containing impurities. The isolation of an hyaluronidase inhibitor which is homogeneous and contains uronic acid has been described by Newman *et al.* (201). A review covering investigations leading up to the isolation of this inhibitor, as well as a review of other hyaluronidase inhibitors, has appeared [Mathews & Dorfman (202)]. A convenient simple turbidimetric assay procedure based on the formation of insoluble complex between hyaluronic acid and cetyltrimethyl ammonium bromide has been developed by DiFerrante (203).

Other polyglycosylases.—Phaff & Demain (204) have shown that a highly purified and apparently enzymically homogeneous yeast polygalacturonase can degrade pectic acid to digalacturonide, in contrast to reports in the literature that a complex of bacterial pectic enzymes may be required for this degradation, [e.g., Kaji (204a)] thus again raising the problem of en-

zyme multiplicities. The course of hydrolysis of most polyglycosidases seems to be characterized by a rapid initial hydrolysis followed by slow degradation to a disaccharide unit. Decomposition of xylan by a crystalline enzyme from bacilli was shown by Inaoka & Soda (205) to be no exception to this rule. Among the fractionation procedures may be mentioned the use of rivanol precipitation and the fairly uncommon crystallization from an organic solvent. It would be of considerable interest to test the cellulase activity of this enzyme in view of the suggestion of Sørenson (206) that the cellulase of *Sporocytophaga myxococcoides* can cleave xylan.

An apparent exception to the above-mentioned rule is the demonstration by Sery & Hehre (207) that an enzyme system from an intestinal bacterium of the *Bacteroides* genus is capable of hydrolyzing dextrans to glucose in contrast to dextranases from other microorganisms. However, it is quite possible that more than one enzyme may be involved here.

Tracey (208) and Clarke & Tracey (209) have investigated in a preliminary manner the occurrence of constitutive chitinase activity in bacteria and in fungi capable of producing N-acetyl glucosamine with a concomitant drop in viscosity. Rose & György (210) describe an enzyme from *Lactobacillus bifidus* strain which can liberate N-acetyl glucosamine from polymers of this compound which appear to act as essential growth factors for this organism.

Transglycosylation

This discussion will be confined in large part to the transglycosylation activity of hydrolytic and similar enzymes and will not consider related enzymes. The status to 1955 of investigations and concepts in this field is the subject of a review by Edelman (211). Although investigations have aided in substantiation of the hypothesis that glycosyl rather than glycoside is involved in these transfers, apparently the simple concept of intermediation of a glycosyl-enzyme complex cannot be applied with the universality hitherto assumed.

Transglucosylases.—The reversibility of the transglucosylation reaction of isomaltose to yield isomaltotriose and glucose by an *Aspergillus oryzae* enzyme has been demonstrated by Pazur (212). The enzyme can catalyze the incorporation of labeled glucose into isomaltose when incubated with nonlabeled isomaltose or with isomaltotriose. In contrast to a similar enzyme from *Aspergillus niger* the *A. oryzae* enzyme cannot synthesize maltose from panose and glucose but can form α-1,3-glucosides from maltose [Pazur *et al.* (213)]. An enzyme preparation from a strain of *Penicillium chrysogenum* can convert maltose into glucose and into two series of oligosaccharides: the isomaltose (dextran) series and the panose. Members of the maltose series are not formed [Saroja *et al.* (214)]. Conversion of -1,4- to -1,6- linkages in the β- as well as the α-configuration of oligosaccharides can occur in view of the finding of Buston & Khan (215) that gentiobiose as well as cellotriose accumulates in a digest of cellobiose incubated with extracts from *Chaetomium globosum.* However, judgment must be deferred until more

evidence is available on the enzymic homogeneity of this and similar preparations which show similar wide specificities. Transcellobiase activity of barley β-glucosidase preparation has also been observed [Manners (152)].

A novel enzymic synthesis of a "branched" trisaccharide from sucrose and lactose was demonstrated by Bailey *et al.* (216) via the action of an enzyme system from *Betacoccus arabinosaceous*. A glucose unit transferred from sucrose apparently by dextran sucrase yielded O-β-D-galactopyranosyl-(1→4)-(α-D-glucopyranosyl-(1→2)-D-glucopyranose. It thus appears from this instance and others previously mentioned that many of the transglycosylation reactions are not strictly specific with respect to the position of the newly formed glycosidic linkage. In the case of dextran sucrase both water and glucose may act as weak glucosyl acceptors, according to Goodman *et al.* (217), on the basis of finding labeled glucose in a digest containing labeled sucrose. Transglucosylation (rather than the transfructosylation, characteristic of leaf enzymes) from sucrose is apparently the mechanism of formation of trisaccharides (mainly melezitose) found in insect-produced honey dew, as found independently by Bacon & Dickinson (218) and by Wolf & Ewart (219).

Specificity studies on branching enzymes (which convert amylose to amylopectin or glycogen via transglucosylation of a portion of the amylose chain to the α-1,6-position) from animal and plants [Larner & Uwah (220)] and from yeast [Manners & Maung (221)] are apparently quite different from the branching enzyme of *Polytomella cocca* in that only the latter enzyme can utilize di- and trisaccharides as substrates.

Other enzymes recently studied in detail in which the mechanism seems to involve transfer of chains of glucose units without changing the resulting linkage (i.e. α-1,4-) include the disproportionating enzyme (D-enzyme) from potatoes and the so-called amylase from *Bacillus macerans*. The latter enzyme, which should be distinguished from a true amylase present in the culture fluid of the microorganism [Schwimmer (222)], has been the subject of a comprehensive study by Cramer & Steinle (223) who propose a reaction cycle for the breakdown of starch in which the cyclic and straight chain dextrins interact with each other via redistribution reactions [Norberg & French (224)] to again yield cyclic dextrin, etc. Peat *et al.* (225) showed by direct comparison that the mechanism of "D-enzyme" action is different from all other known transglucosylations, in that it involves a transfer of two or more glucose units from a maltodextrin to relatively unspecific suitable acceptors, resulting eventually in an equilibrium mixture of maltodextrins (excluding maltose) and glucose. Maltose, which is not a donor, does not appear as a residual fragment in the transglucosylation, indicating that the linkage penultimate to the reducing end of the dextrinases is resistant to scission via "glucosolysis." The relation of "D-enzyme" to the alteration of phosphorylase-produced amylose chains has been discussed by Porter & Rees (226) and by Schwimmer & Weston (227). Summer & French (169) employed the *B. macerans* enzyme to prepare homologous series of singly

branched maltodextrins from isomaltose and from panose, respectively. A spectrophotometric method for distinguishing members of the isomaltose, maltose, and panose series has been developed by Schwimmer & Bevenue (228).

Related but perhaps not strictly analogous to transglucosylation reactions hitherto discussed are the findings of Peat *et al.* (229) that the polymerization of glucose by an enzyme system from an *A. niger* strain resulted in the formation of α- as well as β-disaccharides in contrast to the formation of only β-disaccharides from emulsin.

Transfructosylation.—Investigations on transfructosylations in the past year afford excellent illustrations of the comparative biochemistry of the acceptor specificities as manifested by the predominance of water as acceptor (invertase), on the one hand, and to the action of levan sucrase on the other. Thus, investigation with agave revealed a transfructosylase characterized predominantly by transfer to sucrose to yield oligoglucofructoside with only a small amount of free fructose (transfructosylation to water) being formed [Bhatia *et al.* (230)]. Allen & Bacon (231) found that transfructosylation from sucrose by leaf extracts of higher plants resulted in the formation of two trisaccharides common to the action on sucrose of both yeast and fungal enzymes. But in contrast to the fungal enzymes, these extracts did catalyze the formation of significant amounts of the higher oligosaccharides. Nor was there more than a trace (if any) of C^{14}-labeled glucose incorporated into sucrose during the enzyme reaction, thus throwing some doubt on previous speculations that the leaf invertase may serve as a means of sucrose synthesis in the leaves of higher plants. The action of glucose as an inhibitor of hydrolysis of sucrose by invertase, without inhibiting formation of oligosaccharide intermediates, is apparently distinct from the effect of urea, which equally inhibits both manifestations of yeast invertase action [Krotkov *et al.* 232)].

Hestrin & co-workers (233) have contributed substantially to knowledge of the mechanisms of transfructosylations via the levan sucrase of *Aerobacter levanicum* in particular, and to transglycosylation in general. These workers found that the fructofuranosyl moiety of the trisaccharide raffinose can be transferred via levan sucrase to the anomeric carbon position of glucose and other aldoses to form sucrose and its analogues. Avigad *et al.* (234) have proved that the disaccharide formed with xylose is α-O-xylopyranosyl-β-O-fructofuranoside (xylosucrose). This disaccharide was then used as a tool to elucidate the mechanism of action of other sucrose-transforming enzymes. Thus, xylosucrose is attacked by yeast invertase (a β-fructofuranosidase), but not by dextran sucrase, amylosucrase, or by a β-glucosidase (*Rohferment*). In further studies of levan sucrase action, Hestrin *et al.* (234a) found that in a series of several classes of oligofructosides, those with a β-fructofuranoside group linked to the anomeric carbon of a glucose aldose formed levan and significant quantities of oligosaccharides, free fructose, and free aldose, whereas compounds with the same group

linked to a carbinol carbon did not react. Furthermore, substitution of the fructoside moiety at C-1, C-3, or C-6 also prevented enzyme action. The authors interpreted their findings in terms of the high free energy content of the sucrose-type compounds (fr∼R) as compared with carbinol-linked fructosides with low free energy content (fr<R). The authors propose that the mechanism of levan sucrase action involves the formation of a high free energy enzyme-substrate complex followed by irreversible transfer of fructosyl to a carbinol carbon or to water: fr∼R+enzyme⇌fr-enzyme+R; fr∼enzyme+acceptor→fr<acceptor+enzyme, where the acceptor activity decreases in the order: terminal group of the growing levan molecule; 1-β fructosyl sucrose; sucrose, water. Invertase action represents an apogeal situation: fr∼R+enzyme→fr<enzyme+R; fr<enzyme+acceptor⇌fr<acceptor+enzyme, where water is the most active acceptor. When applied to mold invertase, this theory predicts features common to both levan sucrase and to yeast invertase transfructosylations. Here again we encounter a propinquity to the theme mentioned in the introduction, inasmuch as the action of the mold enzyme system may be alternatively interpreted as owing to the resultant action of a multiplex system showing varying degrees of specificity.

The implication of this theory that the free energy relations are such that the levan sucrase action is strictly irreversible is not strictly in accord with the results of Peaud-Lenoël (234b) with levan sucrase of *B. subtilis*, and confirmed by Hestrin *et al.* (234a) with the *A. levanicum* enzyme, although the magnitude of the free-energy term may be such as not to obviate the main conclusions of Hestrin and co-workers. Further studies by Feingold *et al.* (234c) suggest that this magnitude depends upon the specific carbinol acceptor involved. This idea can be illustrated in general for other transglycosylases. For instance the synthesis of amylose from sucrose is reversible, whereas that of dextran is essentially irreversible. This would also explain why some transfructosylation to secondary carbinol positions occurred from the fr∼enzyme complex formed from sucrose by levan sucrase, but does not occur from the fr<enzyme complex formed with yeast invertase. However, the main oligosaccharide formed from levan sucrase action was found to be 1^F-β-fructosyl fructose.

Transgalactosylases. Some interesting papers on transgalactosylation have appeared in the last year. Zilliken *et al.* (235) report a nearly selective synthesis of microbiologically active 4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine when intact cells of a strain of *L. bifidus* are incubated with lactose and N-acetyl glucosamine, whereas crude lactase preparations from the same microorganism yielded a mixture of 4- and 6-O-β-D-galactosides. The latter galactoside was synthesized both chemically and enzymatically (*E. coli* source) by Kuhn *et al.* (236). The *E. coli* enzyme preparation could also form 6-O-β-D-galactopyranonsyl-D-glucose via transgalactosylation to glucose from β-phenyl D-galactoside. The 3-O-β-D-galactosyl-N-acetyl-O-glucosamine component of oligosaccharides present in human milk [Kuhn *et al.* (237)]

was found as a component of mixtures of the galactosides of N-acetyl glucosamine formed via lactase of mammalian origin [Allesandrini *et al.* (238)].

Transglucuronosylation.—Evidence that liver β-glucuronidase can catalyze transfer of glucuronosyl groups from phenolphthalein ester to propylene glycol and other alcohol has been presented by Fishman & Green (239).

Further indirect evidence for transglucuronosylation to polyalcohols is afforded by the observations of Nayyar & Glick (240) that these alcohols increase β-glucuronidase activity. In a study of the kinetics of the effect, it was found that the inhibition produced by excess substrate (phenolphthalein glucuronide) was not influenced by ethylene glycol, in that there was no change in the dissociation constant of a presumed ES_2 complex, but the K_m value was decreased. Although the authors suggest that this indicates an apparently increased dissociation constant of enzyme-substrate complex and that the effect may be explained on the basis of an increase in k_3, the speculation that transglucuronosylation is involved may be more realistic, especially in view of the previously discussed paper of Fishman & Green (239).

Hyaluronidase.—It is now clear that extensive transglycosylation of oligosaccharide accompanies the enzymatic hydrolysis of hyaluronic acid. Thus Weissman (241) showed that when individual oligosaccharides isolated from testicular hyaluronidase action on hyaluronic acid are employed as substrates for this enzyme, the reaction is characterized by rapid transglycosylation of disaccharide units accompanied by a slow hydrolysis. Hoffman *et al.* (242) showed that the position of the hexosamidinic linkage in these oligosaccharides remains unchanged during transglycosylation with testicular hyaluronidase. Furthermore, they showed that when hyaluronate and chondroitin sulfate are employed simultaneously as substrates for this enzyme, hybrid oligosaccharides are formed which contain disaccharide repeating units from both polysaccharides.

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PROTEOLYTIC ENZYMES[1,2]

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The reviewers have had available a wide choice of deprecating remarks by their predecessors concerning the scope and thoroughness of coverage of this type of review. Fraenkel-Conrat (3) has in recent years come perhaps closest to our own feelings on the subject, and with minor modification to fit the subject matter we wish to echo the statements of his first paragraph.

The period covered by this review has brought forth a number of papers wherein new enzymes have been characterized only by their ability to split esters such as tosyl.Arg.OMe. It is very likely that many of these enzymes would be equally capable of splitting peptides even though to date this has not been specifically demonstrated. Reports dealing with such borderline cases have generally been included in this review. Also, of the large number of papers dealing with the proof of amino acid sequence in proteins (other than proteolytic enzymes) by means of controlled enzymatic hydrolysis, only those were chosen which appeared to have a bearing on the substrate specificity of proteolytic enzymes.

For many of the enzymes covered in this review, rather extensive descriptions of properties and methods of preparation have been compiled (4). Several aspects of the mechanism of action of proteolytic enzymes have been summarized and discussed in the symposium *The Physical Chemistry of Enzymes* (5).

Exopeptidases

Carboxypeptidases.—The isolation of the inactive precursor, procarboxypeptidase, from beef pancreas in a state of 95 per cent purity has been described (6, 7). It differs markedly in some of its physical properties (isoelectric point = approx. pH 4.2; sedimentation constant = 5.87 S; molecular weight = 96,000) from carboxypeptidase into which it is converted specifically by trypsin. In extension of a preliminary note (8), a more detailed account of the evidence which designates carboxypeptidase as a zinc-metalloenzyme has been published by Vallee & Neurath (9).

Nonspecific side reactions in the use of recrystallized carboxypeptidase

[1] The survey covered by this review includes the literature available to the authors up to October 1st, 1956.

[2] Amino acid residues have been abbreviated as originally proposed by Brand *et al.* (1) and their derivatives according to the convention used by Schwert (2). Unless otherwise stated, amino acids, as mentioned, are of the L-form. The following additional abbreviations have been used: DENP for diethyl-*p*-nitrophenylphosphate; DFP for diisopropylphosphorofluoridate; DIP for diisopropylphosphoryl; DNFB for 2,4-dinitrofluorobenzene; DNP for 2,4-dinitrophenyl; EDTA for ethylenediamine tetra-acetic acid.

preparations for analytical purposes have been found previously to be attributable to admixtures of trypsin and chymotrypsin. More recently, Steinberg (10) added to this list a proteolytic enzyme, possibly of bacterial origin, with properties similar to subtilisin. The effect of these contaminations could be eliminated by pretreatment of the enzyme preparation with DFP. A new pancreatic ("basic") carboxypeptidase has now been described by Folk (11) as an impurity in insufficiently recrystallized "carboxypeptidase." This enzyme appears to act specifically on peptides containing a basic C-terminal amino acid; thus, it readily attacks bz.Gly.Arg and bz.Gly.Lys (but not bz.Gly.Lys.ONH_2). Like Anson's carboxypeptidase, it occurs in pancreas as a trypsin-activatable proenzyme. Specific inhibitors are δ-aminovaleric and ε-aminocaproic acid.

Leucine aminopeptidases.—The use of L-leucylnaphthylamide as a substrate in the histochemical localization of aminopeptidases has been described (12). Hill & Smith (13) have extended their studies on substrate specificity to include amino acid amide derivatives of 2-aminofluorene, 4-aminobiphenyl and 4,4′-diaminobiphenyl. When various amino acids were linked to these carcinogens, the rates of hydrolysis of the resultant peptides, while low, were in the same relative order as for the corresponding unsubstituted amides. Waldschmidt-Leitz & Mindemann (14) have described the use of purified pancreatic leucine aminopeptidase in the determination of N-terminal residues of 13 proteins. The extensive degradation of oxidized and unoxidized insulin by means of prolonged incubation with this enzyme has been demonstrated in a preliminary note (15).

Dipeptidases and other exopeptidases.—Vescia (16) has compared the relative activity of hog kidney leucine aminopeptidase [partially purified through step IV of Spackman *et al.* (17)] against Leu.Gly, Gly.Leu, and Gly.Gly as influenced by addition of Mn^{++} or Co^{++}. On the basis of mixed substrate experiments and the constancy of relative activities through the various stages of purification the author claims that all three substrates are acted upon by the same enzyme. The results are in agreement with earlier ones by the same author on placental aminopeptidase preparations (18); however, disagreements with the findings of Smith & Spackman (19) on the specificity of highly purified aminopeptidase are probably more apparent than real since the enzyme preparation used is quite heterogeneous (17), and any implied identification of this enzymatic activity with leucine aminopeptidase appears unwarranted.

Fleisher (20) has likewise ascribed the hydrolysis of Gly.Leu, Leu.Gly, and Gly.Tyr by a purified preparation from leucocytes and lymphocytes to a single enzyme. This preparation, when acting on Gly.Leu, was maximally activated by preincubation with low concentrations of Zn, and inhibited by EDTA or 10^{-3} *M* Ca; this inhibition could be maximally overcome by Zn only. The hydrolysis of Leu.Gly and Gly.Tyr by this enzyme preparation was stimulated to a much smaller extent by Zn than that of Gly.Leu and

showed different pH optima. Therefore, no definite conclusion is possible as to whether only one enzyme is responsible for these hydrolyses.

Attempts have been made to correlate the influence of hormones and vitamins on protein metabolism with their effects on individual enzymes. According to Schwartz and co-workers (21), surviving diaphragm slices from adrenalectomized rats exhibit a significantly lower activity against Gly.Gly than those of controls; intraperitoneal administration of cortisone increases this activity. Neither cortisone nor hydrocortisone had any significant *in vitro* effect. Along similar lines, the increased protein catabolism in vitamin-E deficiency has been related to the finding (22) of a markedly increased activity of muscle extracts and homogenates against Gly.Gly (in presence of Co^{++}) and Gly.Leu in avitaminosis-E.

Rabin (23) and Rabin & Crook (24) have made the important point that in kinetic considerations of interaction between metal-activated enzyme (E), metal (A), and substrate (S), the effect of A-S interaction has frequently been disregarded. This becomes especially serious when such interaction results in highly stable chelates. Thus, the value of the dissociation constant for the system Co^{++}:glycylglycine dipeptidase is 1.1×10^{-7} M when this is taken into account, in contrast with the previous value of 2.8×10^{-5} M (25). The possibility exists that reaction mechanisms other than combination of EA with S may be involved in such cases: for instance, combination of A with ES, or preformation of SA which then acts as the "true" substrate.

Endopeptidases

Pepsin.—The molecular weight of pepsin has been reinvestigated by light scattering procedures and the experimental mean estimated at $34{,}800 \pm 870$ (26). The agreement of this value with earlier estimates [e.g. (27)] is regarded as fortuitous because of occasionally considerable contamination of most preparations with nonprotein material. However, it should still be considered as an upper limit because of the extreme susceptibility of pepsin to surface denaturation and consequent formation of large-size particles; molecular weights as low as 32,500 were obtained when special precautions were taken to keep denaturation at a minimum. Dieu (28), likewise, reports a molecular weight of 35,000 at pH 2.55 on the basis of light scattering experiments, but, by sedimentation and diffusion studies, values of 27,000–28,000 at pH 5.2, 7.2, and 8.3. At least in part, these lower values result from significantly higher diffusion constants than are usually found. Aggregation does not appear to be associated with surface denaturation invariably, since increased viscosity and decreased rate of sedimentation are also found in monomolecular layers of pepsin (29, 30). This is in agreement with the results of Edelhoch (31), according to which the inactivation of pepsin in the pH range 6.4 to 7.1 effected a decrease in light scattering, an increase in viscosity and levorotation, and an acid liberation to the extent of 5.6 moles per mole of enzyme; the rate of these changes paralleled the rate of loss of

peptic activity. Similar changes are observed during ultraviolet irradiation of pepsin (32) which results in a marked increase of intrinsic viscosity (from 5.16 to 18.5) and of axial ratios (from 4.2 to 12.4),[3] with loss of enzymatic activity but without noticeable change in molecular weight. It would appear, therefore, that at least the initial stages in denaturation of pepsin are characterized by a change in the shape and structure of the molecule, presumably associated with breakage of hydrogen bonds. The interesting observation (32) that 3,4-dihydroxyphenylalanine can be isolated from irradiated pepsin after hydrolysis would be analogous to the findings of Haas *et al.* (34) on pepsin after treatment with tyrosinase.

In a preliminary communication, Williamson (35) reports that crystalline pepsin, after autolysis at pH 5 and 4°C. or 37°C. with dialysis for 48 hr., retained all its enzymatic activity in the nondialyzable component; this result appears to be in contradiction to Perlmann's (36) previous findings. Williamson further reports the liberation of phenylalanine and of leucyl peptides during autolysis at pH 5, and, in addition, of methionine at pH 3.5. The N-terminal sequence, on the basis of stepwise degradation and in a modification of that previously reported, is now stated to be Leu.Gly.Asp.-Asp.His.Glu (37). The presence of the single phosphate group of pepsin in a diester linkage, previously indicated (38), has been confirmed by Perlmann (39). Since pepsin consists of a single chain, it is concluded that this phosphorus serves to form a cyclic structure.

The interaction between pepsin and lysozyme produces an adduct with minimum solubility and isoelectric point at pH 7.3 (40). Remarkably enough, a single boundary is formed when equal weights of the two constituents in solution are subjected to ultracentrifugation; this would indicate an adduct containing about two molecules of lysozyme per mole of pepsin.

Kassell & Laskowski (41) have determined pepsin manometrically, using tosyl.Arg.OMe as substrate. In the same paper the widely different effect of pepsin on six natural trypsin inhibitors is reported. Baker's previously described observations (42) on the hydrolysis of ac.Tyr.Tyr and ac.Phe.Tyr are discussed in concurrently published notes by Green & Baker (43). The inhibitory power of ac.Phe on the hydrolysis of ac.Phe.Phe is higher than previously reported if fresh mixtures of enzyme and inhibitor are used, rather than mixtures prepared and stored overnight, as in the previous procedure; this might indicate a deacylating action of pepsin on ac.Phe.

Chymotrypsin.—In a continuation of earlier studies of the activation of chymotrypsinogen, Rovery *et al.* (44) present evidence for a new intermediate ("α_1-chymotrypsin") in the slow activation of chymotrypsinogen by trypsin. This form contains N-terminal isoleucine and threonine; the latter is gradually replaced by alanine to give α-chymotrypsin. Although the action of δ-chymotrypsin on chymotrypsinogen does not result in an increase in

[3] The author quotes differing results in the table and in the text. Recalculation of his data indicates the above figure to be correct. Also, by comparison with data quoted by Cohn & Edsall (33), the axial ratio a/b rather than b/a appears to have been calculated.

active enzyme, significant quantities of N-terminal threonine and alanine are produced. Further studies of the action of α-chymotrypsin on chymotrypsinogen in the same laboratory (45) indicate that two new forms of chymotrypsinogen ("Neo-Chymotrypsinogens") are produced, both with C-terminal tyrosine but containing N-terminal alanine and threonine, respectively. The C-terminal sequence appears to be (Lys, Leu).Tyr. The new forms of chymotrypsinogen could be fully activated by trypsin although the activation was slower after carboxypeptidase treatment. Dreyer & Neurath (46) have reported in more detail the results of their study on the formation of Ser.Arg during the conversion of π- to δ-chymotrypsin. Dreyer, Wade & Neurath (47) find, in confirmation of Egan *et al.* (48, 49), that the products of both rapid and slow activation of chymotrypsinogen are electrophoretically heterogeneous. Uncrystallized DIP-π- and δ-chymotrypsin were found to be monomeric at pH 3.86 but showed dimeric behavior at pH 7.5. Bronfenbrenner *et al.* (50) have further investigated the previous finding of Abrams & Jacobson (51) that crude preparations of subtilisin can convert chymotrypsinogen to chymotrypsin; by the use of crystalline subtilisin as the activator, the intermediate appearance of a new form of chymotrypsinogen, activatable by trypsin, was demonstrated. Chromatographic studies of the slow activation of chymotrypsinogen by Sakota (52) revealed two heterogeneous fractions, of which the second appeared to contain α-chymotrypsin. Sakota *et al.* (53) reported a new crystalline form of chymotrypsin which yielded α-chymotrypsin on repeated recrystallizations plus short storage; from the method of preparation, it would appear that this form may be related to that of Rovery *et al.* (44). Raacke (54) found α-chymotrypsin and chymotrypsinogen to be heterogeneous on electrophoresis on starch.

The foregoing reports emphasize the complexity of events associated with the activation of α-chymotrypsinogen. Many of the postulated "intermediates" may well be the result of autolytic processes or changes in the physical state of the protein(s), and their importance in the main pathway of activation is largely unknown at present.

Several laboratories have attempted to extend the peptide sequences associated with the serine residue which is phosphorylated during the inactivation of chymotrypsin by organophosphorus compounds. Turba & Gundlach (55) have isolated two phosphorus-containing peptides from DIP-chymotrypsin which contained (*a*) aspartic acid, serine, glycine, and glutamic acid; and (*b*) serine, glycine, glutamic acid, and alanine respectively. These workers also reported the sequence Asp.Ser.Gly, thus confirming Schaffer *et al.* (56). In continuation of earlier work, Schaffer and his coworkers (57) have established the sequence Gly.Asp.Ser.Gly for one of the labeled peptides isolated by them. Acid hydrolysis of Sarin (isopropyl methylphosphonofluoridate)-inactivated chymotrypsin yielded a peptide containing Gly,Asp,Ser,Glu,Ala, and Val. The peptide appeared to have the N-terminal sequence Gly.Asp and either alanine or valine at the C-terminus. The composition of a larger labeled peptide, isolated from a papain digest of Sarin-inactivated chymotrypsin, was described by the same authors. Ooster-

baan *et al.* (58) have also obtained a peptide from DIP-chymotrypsin having the sequence Gly.Asp.Ser.Gly and containing in addition proline, leucine and possibly a third glycine residue. While the results reported by the latter two laboratories (57, 58) agree on the sequence Gly.Asp.Ser.Gly, they are not consistent with respect to the other residues.

Meedom (59) has obtained three peptide fractions from performic acid-oxidized chymotrypsin and DIP-chymotrypsin. One of the peptides, containing 13 amino acids, had N-terminal cysteic acid and C-terminal leucine; a second peptide, containing 22 amino acids, had N-terminal alanine, and a third large peptide had N-terminal leucine or isoleucine and C-terminal tyrosine.

Gross & Egan (60) have reduced α-chymotrypsin with sulfite in the presence of *p*-chloromercuribenzoate. After complete reduction, all enzymatic activity was lost and 70 per cent of the material was dialyzable and soluble in 20 per cent trichloroacetic acid. Wilcox & Chervenka (61) found the α-amino group and all 13 ε-amino groups of chymotrypsinogen to react with carbon disulfide and with N-methyl isourea. All derivatives resulting from these reactions were converted into active enzymes by trypsin, thus demonstrating the nonessential character of the amino groups, with the possible exception of the one produced during activation.

The inactivation of chymotrypsin by 1-dimethylaminonaphthalene-5-sulfonyl chloride has been reported by Hartley & Massey (62). The reaction was stoichiometric and produced an unstable fluorescent conjugate. Excess dye caused higher degrees of labeling and a change in color. Chymotrypsinogen and DIP-chymotrypsin also formed conjugates with the dye but with lower absorption maxima than those of chymotrypsin. Of various amino acids and derivatives tested, only bz.His.OMe formed a conjugate having spectral properties similar to those of the protein after reaction with an equimolar amount of dye. The same authors (63) have reported on the mode of reaction of chymotrypsin with a large excess of DNFB, under various experimental conditions. DNFB was found to react with one alanine, one histidine and six lysines of DIP-chymotrypsin at room temperature. Interestingly enough, isoleucine, whose N-terminal position in DIP-chymotrypsin is well established, reacted only very slightly under these conditions. Prolonged reaction of chymotrypsin with DNFB at −3°C. in 17 per cent ethanol was reported to increase K_m without affecting V_{max} when enzymatic activity was measured in the presence of methanol. However, this finding was subsequently modified by Hartley (64) who observed a decrease in V_{max} without change in K_m when the activity was measured in the absence of methanol. Conditions have been found by Whitaker & Jandorf (65) for the inactivation of chymotrypsin by DNFB which involved minimal labeling of the protein. At pH 10.66, with a molar ratio of DNFB to chymotrypsin of 1.2, over 50 per cent inactivation of chymotrypsin occurred with less than one mole of DNFB reacted per mole of protein.

Hammond & Gutfreund (66) have studied the influence of pH on the chymotryptic hydrolysis of ac.Phe.OEt. Their results indicate that the

initial formation of enzyme-substrate complex is independent of the subsequent reaction at the catalytic site. The ionization constant of the catalytic site, $pK_i = 6.85$, was considered to be consistent with that of an imidazole group. Cunningham & Brown (67), in a study of the effect of pH on the hydrolysis of ac.Tyr.OEt and ac.Try.OEt by α-, β-, and γ-chymotrypsins, found that K_m is constant in the range pH 6 to 8 but increases at higher pH values; V_{max} increases over the same pH range to a maximum value at pH 8.7. The results of both laboratories are in good agreement and also indicate that an ionizable group with an approximate pK of 9, essential for the formation of the enzyme-substrate complex, is present in chymotrypsin. The probable presence of an anionic site on chymotrypsin, involved in its combination with negatively charged inhibitors, as previously postulated in several laboratories (68, 69, 70), has also been shown by Cunningham & Brown (67).

In two reports on the mechanism of chymotrypsin-catalyzed reactions, Gutfreund & Sturtevant (71, 72) have used a stopped-flow technique to study the rates of reactions involved in the hydrolysis of *p*-nitrophenyl acetate, 2,4-dinitrophenyl acetate, and ac.Tyr.ONH_2. The rate of formation of the enzyme-substrate complex was found to be too fast to be measured by their technique. In the hydrolysis of the nitrophenyl acetates, the liberation of the phenol which followed first order kinetics was found to be much faster than the subsequent deacetylation. The rate of formation of 2,4-dinitrophenol was dependent on the ionization of a group in chymotrypsin having a pK of 6.7 whereas the release of acetate was dependent on a group with a pK of 7.3. It was suggested that the first catalytic step involves an acylation, possibly on a serine hydroxyl, and that this leads to a shift in the pK of the catalytic group. The results obtained by Laidler & Barnard (73) in a continuation of earlier studies on the kinetics of hydrolysis of methyl β-phenylpropionate by α-chymotrypsin show that V_{max} has an optimum value near pH 8, in contrast to the results obtained by other laboratories (66, 67). Further work will be needed to clarify this point.

Other evidence concerning the role played by an imidazole residue in the reactions of chymotrypsin with substrates and inhibitors, reviewed by Jandorf *et al.* (74), has accumulated during the last year. Thus, the rate of reaction of α-chymotrypsin with DENP has been found by Hartley (64) to depend on the ionization of a group of pK 6.6 in the enzyme.

In a seemingly contradictory report, Dixon *et al.* (75) find no difference in light absorption between chymotrypsin and acetyl-chymotrypsin[4] in the range 230 to 600 mμ. It was stated by the authors that if an acetyl-imidazole bond had formed it would have been detectable as a change in absorption at 245 mμ. However, it should be noted that N-methyl imidazole is almost as

[4] Acetyl-enzyme refers to the addition product of a substrate-derived acetyl group to the catalytically active site of the enzyme. The stable derivatives of enzymes obtained by the action of acetylating reagents on groups other than the active site will be referred to as acetylated enzymes.

effective as imidazole in catalyzing the hydrolysis of *p*-nitrophenyl acetate (76) and of DFP (77).

The effect of sucrose on the hydrolysis of chloroacetyl Tyr.ONH_2 by α-chymotrypsin has been studied by Shine & Niemann (78). K_m was unaffected but V_{max} was increased 24 per cent in 0.5 *M* sucrose. These results are to be contrasted with those found for the action of methanol on chymotrypsin (79), which affects K_m without changing V_{max}. Wu & Laskowski (80) report that calcium ions increase the activity of B-chymotrypsin and stabilize both α- and B-chymotrypsin. Vaslow (81) has given a detailed report concerning the α-chymotrypsin-catalyzed oxygen exchange between water and N-acetyl-3,5-dibromotyrosine. Oxygen exchange reactions catalyzed by α-chymotrypsin have also been studied by Bender & Kemp (82). Benzoyl-L-phenylalanine, acetyl-L-tryptophan, benzoyl-D-phenylalanine and β-phenylpropionic acid, all labeled with O^{18} in the carboxyl group, were used as substrates. Only benzoyl-L-phenylalanine and acetyl-L-tryptophan showed an exchange of oxygen.

Balls & Wood (83) report more details and further work on the formation and properties of acetyl-chymotrypsin.[4] In the presence of ethanol, acetyl-chymotrypsin formed ethyl acetate so that chymotrypsin may be regarded as a transacetylase which transfers acetyl groups from nitrophenol to ethanol. Further work from the same laboratory (84) indicates that other aliphatic alcohols may likewise act as acetyl acceptors, the rate of transfer increasing with chain length up to C_6. Methanol is an exception in that it reacts twice as fast as ethanol. Transesterification between butyl alcohol and Tyr.OEt was also demonstrated.

An x-ray study of crystalline α-chymotrypsin by Bluhm & Kendrew (85) indicated a molecular weight of $25{,}000 \pm 800$, assuming four molecules per unit cell in the crystal. Osmotic pressure and light scattering measurements were used by Bartl & Sedláček (86) to show that substrate association with chymotrypsin is irreversibly suppressed after inactivation of the enzyme with DFP. Chloramphenicol suppressed the association reversibly.

McLaren & Estermann (87), in a study of the hydrolysis of denatured lysozyme adsorbed on kaolinite by chymotrypsin, trypsin and acetylated trypsin,[4] have shown that proteolysis is brought about chiefly by adsorbed enzyme. The rate of hydrolysis in the adsorbed state was comparable to that occurring in solution. These results are analogous to those found by Trurnit (88, 89) in a study of the chymotryptic hydrolysis of bovine serum albumin adsorbed on chromium-coated glass slides.

Booman & Niemann (90) have proposed a statistical method for evaluating the initial velocities of enzyme reactions and applied it to the hydrolysis of Try.ONHOH by α-chymotrypsin. The method is independent of any assumed reaction mechanism.

Trypsin.—Dixon *et al.* (91) have obtained two labeled peptides from a chymotryptic hydrolysate of trypsin which had been inactivated with C^{14}-labeled DFP. After reaction with DNFB followed by acid hydrolysis, one of the peptides yielded cysteine, aspartic acid, glutamic acid, glycine, serine,

alanine, proline, and valine but no DNP derivative. The other peptide contained the same amino acids and in addition ϵ-DNP-lysine. Lysine was probably C-terminal since α,ϵ-di-DNP-lysine was not found. Oosterbaan *et al.* (58) have obtained a peptide from DIP-trypsin which had the composition Asp,Ser,Pro,Val,$Gly_{(2\text{ or }3)}$. Three phosphonyl peptides, obtained from an acid hydrolysate of Sarin-inactivated trypsin by Schaffer *et al.* (57), had the following structures: $Ser(CH_3PO_3)$.Gly, $Asp.Ser(CH_3PO_3)$, and Asp.Ser-(CH_3PO_3).Gly. Thus the sequence Asp.Ser.Gly, which is possibly an essential component of the active site, is identical in trypsin and chymotrypsin.

The activation of trypsinogen by enterokinase has been studied by Yamashina (92). N-terminal valine was found throughout the activation; the appearance of N-terminal isoleucine paralleled that of tryptic activity, indicating a mechanism which may be identical with that of the activation by trypsin (93, 94).

In an attempt to obtain evidence regarding the formation of an unstable, phosphorylated intermediate during the inhibition of trypsin by DFP, Dixon & Neurath (95) dialyzed the freshly inactivated enzyme in the presence of 8 *M* urea; however, no evidence for an unstable phosphoryl group was found. Moreover, no significant incorporation of phosphorus occurred when trypsin was allowed to react with DFP in the presence of 8 *M* urea. The latter finding is not inconsistent with results obtained on the reaction of Sarin with trypsin in the presence of high concentrations of urea (96). Under these conditions, phosphorylation of the enzyme did occur but at a much slower rate than in the absence of urea.[5] Viswanatha & Liener (97, 98) concluded from their study of the reaction of DENP with trypsin in high concentrations of urea that the normal site of phosphorylation is distinct from the catalytic site. This view was based on their finding that phosphorylation occurred without loss of activity on dilution and their belief (99) that trypsin retains its activity in 8 *M* urea. In contrast with these results, Harris (100) and Michel (96) showed that trypsin was reversibly inactivated in 6.6 to 8 *M* urea and that the results quoted above were due to the decrease in urea concentration prior to measurement of activity; in an addendum to their most recent paper, Viswanatha & Liener (98) have accepted this explanation also. Harris & Hartley (101) have been unable to demonstrate any reaction of DENP with trypsin in 6.6 *M* urea; the discrepancy between their results and those of the Minneapolis laboratory has not been resolved to date.

The activities of the fast and slow components of trypsin, obtained by electrophoresis in the presence of calcium ions, with hemoglobin, bz.Arg.OEt, and tosyl.Arg.OEt as substrates, were determined by Timasheff *et al.* (102). In different preparations both the fast and "enriched" slow components showed essentially the same relative activities as the original trypsin. The stabilizing effects of acetylation and calcium ions on trypsin activity have

[5] This slow reaction may correspond to that shown to occur between organophosphorus compounds and many proteins, including nonenzymatic ones (74), and would therefore not be concerned with catalytically active sites.

been found to be additive by Terminiello *et al.* (103). The activity of acetylated trypsin[4] in the presence of calcium ions was maintained for two days at 25°C. and pH 8.6 even though an increase in free amino groups was observed. Chemical modification of trypsin, leading to loss of enzymatic activity, has also been achieved with bromoacetate, a reagent which reacts with amino, phenolic, and imidazole groups [Korman & Clarke (104)]. Yon (105, 106) finds that calcium reduces the rate of tryptic hydrolysis of lactoglobulin by combining with the substrate. Ammonium ion completely inhibits hydrolysis of lactoglobulin at pH 7 to 7.7 but has little effect at pH 8.3; casein and hemoglobin hydrolyses are not significantly affected by ammonium ion.

Various papers dealing with the specificity of trypsin for synthetic and protein substrates and with kinetic measurements of the resulting hydrolyses have appeared. In a logical extension of earlier work on carbon-carbon bond splitting by chymotrypsin (107), Shapira & Doherty (108) report that trypsin slowly catalyzes the splitting of ethyl 3-keto-δ-amino-octanoate to yield ϵ-amino caproic acid. Zeller *et al.* (109) have used a manometric method to study the hydrolysis of ester substrates by trypsin. Bz.His.OMe was found to be hydrolyzed but at only 1/50 of the rate of bz.Arg.OEt. Levin *et al.* (110) have investigated the hydrolysis and transpeptidation reactions of lysine peptides by trypsin. They conclude that a free α-carboxyl group adjacent to the sensitive petide bond inhibits the catalysis.

The low activity of trypsin toward chymotrypsin substrates, which had been thought to be due to an impurity of chymotrypsin in trypsin preparations, has been shown by McFadden & Laskowski (111) to be a property of trypsin. This finding may have an important bearing on the interpretation of structural studies with trypsin.

Forrest *et al.* (112) have studied the trypsin-catalyzed hydrolysis of bz.Arg.ONH_2 by a calorimetric method. In contrast with several earlier authors they find the hydrolysis to follow first order kinetics to within 90 to 95 per cent of completion, with substrate concentrations up to 0.06 *M*. Hultin & Lundblad (113), using a viscosimetric method, found K_m for the tryptic hydrolysis of gelatin to be in the range of 0.01 to 0.03 per cent. Heat-denaturated serum albumin, upon standing, becomes progressively more resistant to tryptic hydrolysis [Gorini & Audrain (114)]. This effect is brought on immediately by hydrogen peroxide, and is prevented by cysteine; as a result, the authors conclude that a loss of free sulfhydryl groups by autoxidation in the denatured substrate is responsible for their findings.

Hultin (115) has adopted the hypothesis of Kavanau (116) that the failure of enzyme-catalyzed reactions to follow the Arrhenius law at low temperatures is due to reversible inactivation. A quantitative treatment of this hypothesis was applied to the tryptic hydrolysis of bz.Arg.ONH_2.

A value of 21,000 was found for the molecular weight of trypsin by Sedláček & Bartl (117) by the use of osmotic pressure and light-scattering measurements.

Papain.—Hill & Smith (118), in continuation of their interesting studies on the degradation of mercuripapain by leucine aminopeptidase, now report

the liberation of at least 120 amino acids from the enzyme without loss of enzymatic activity. A peptide containing 27 amino acids, including two $CySO_3H$ residues, was isolated from a tryptic hydrolysate of oxidized mercuripapain by Kimmel & Smith (119). The peptide contained neither arginine nor lysine and was therefore probably C-terminal. In the same study, active papain was inactivated with one mole of iodoacetamide prior to oxidation and hydrolysis. A new peptide was then recovered in place of the one obtained from untreated mercuripapain. Since it is probable that iodoacetamide had reacted with the active site, it was concluded that the latter must be near the C-terminal end of the molecule, a conclusion which is supported by the degradation studies with leucine aminopeptidase.

Kimmel *et al.* (120) have found that only six of the eight atoms of sulfur in papain can be accounted for as half cystines. The remaining two sulfur atoms cannot be derived from methionine or from sulfate groups. Hydrolysis of papain with acid, following oxidation by performic acid, yielded the following peptides, which account for four of the six possible cysteic acids: $Asp.CySO_3H$, $CySO_3H.Asp$, $Ser.CySO_3H$, $CySO_3H.Gly$, $CySO_3H.Gly.Gly.Asp$, and $Val.CySO_3H.Gly(Pro,CySO_3H)$. From the fact that both reduced and dialyzed crystalline papain combine with the same number of equivalents of *p*-chloromercuribenzoate or *p*-chloromercuribenzene sulfonate, Smith *et al.* (121) conclude that dialyzed papain does not contain any disulfide bridges unless mercurials can reduce such bonds, a reaction which they consider to be unlikely. However, it should be noted that Gross & Egan (60) have shown that *p*-chloromercuribenzoate can hydrolyze disulfide bonds in chymotrypsin.

Johnston (122) has published further details concerning the thiolesterase activity of papain. Tollin *et al.* (123) have studied the papain-catalyzed reactions with aniline and glycinanilide of benzoyl dipeptides containing glycine. Transamidation occurred when glycine was adjacent to the benzoyl group, and addition when glycine was in the terminal position.

The formation of acyl lysine anilides from α,ϵ-N-diacyl lysines by papain has been studied by Murachi (124). This stereospecific reaction was applied to the resolution of acyl-D,L-lysines (125). The determination of papain activity by a milk-clotting procedure has been described by Gawron & Draus (126).

Enterokinase.—The further purification of this enzyme has been described by Yamashina (127); the final preparation, estimated to be about 80 per cent pure on the basis of electrophoresis and ultracentrifugation, has an activity of 2,600 Kunitz units (128) per mg. Its carbohydrate constituents (nearly 30 per cent of the dry weight) include fucose, mannose, galactose, glucosamine, and galactosamine.

Collagenases and elastases.[6]—A colorimetric method for the determina-

[6] These two classes of enzymes, if indeed there are two, are considered together deliberately. Previous workers have considered collagen and elastin as not only structurally but also biochemically separate entities. However, the recent histological demonstration by Burton *et al.* (129) of a conversion of collagen into elastin, on incu-

tion of elastase activity, based on the release into solution of color from orcein-stained elastin has been described (130). While this method suffers from the disadvantages of a heterogeneous reaction (the substrate being insoluble) it appears to be reproducible under controlled conditions. The pH optimum of this enzyme is 8.7 to 9.2, in agreement with results by Hall & Gardiner (131). The digestion of collagen by pancreatic fistula juice (132) cannot be ascribed to the presence of chymotrypsin, carboxypeptidase (both inactive against collagen at pH 7 to 8) or of trypsin (the activity of which is lower than that of unpurified fistula juice). Evidence has been presented that elastase is produced in the α-cells of dog pancreas (133) in the form of a precursor ("proelastase") which is activated by trypsin (134). This latter finding raises the possibility that the reported (135) inhibition of elastase by plasma trypsin inhibitor (or a plasma protein with very similar properties) might in reality be an effect on the activation process rather than on the active enzyme.

A definite description of the processes responsible for the solubilization of collagen and elastin will have to await the purification of the enzymes involved. It appears at this time as if proteolysis represents only a part of the process, which involves mucolytic enzymes of pancreatic origin as well. A preliminary description of two such enzymes, termed "elastomucoproteinase" and "collagen mucoproteinase" has been furnished by Banga & Baló (136).

Plasmin (*fibrinolysin*).—Human plasmin has been partially purified after activation of plasminogen with streptokinase (137). The activity of whole activated plasma, as measured by proteolysis of casein or hemoglobin, shows a pH optimum of 7.5 to 8.6 [Meyers & Burdon (138)]. The validity of the kinetic evaluation of this proteolytic action is made doubtful by the demonstration, through dilution experiments, of dissociable inhibitor(s) in their enzyme source. Antifibrinolytic activity of plasma has likewise been demonstrated by others, and the multiple nature of the inhibitory action appears to be well established. Thus, Norman (139) reports inhibition to occur in two stages with different rates; likewise, Siegel *et al.* (140) have purified an inhibitor from human plasma Fraction IV-1, which, in contrast with Schulman's (141) factor, is insoluble in trichloroacetic acid and has no antitrypsin activity.

Considerable purification of the plasminogen activator from human urine ("Urokinase") has been reported by Ploug & Kjeldgaard (142). The factor is a protein of considerable thermostability, and is probably identical with that described by Astrup & Sterndorff (143). The activator preparation is reported to hydrolyze tosyl.Arg.OMe, Lys.OEt, and casein; however, the fact that "high concentrations" of activator are required might indicate a

bation with buffers, proteolytic enzymes, or other chemicals, and the appearance of collagen fibrils in elastin under different conditions of incubation, may cast doubt on the reality of such a distinction. The authors of this review do not feel themselves in a position to judge the validity of these histological findings; the designations "collagen," "elastin," etc., used in the papers to be reviewed have therefore been retained.

nonspecific side reaction of urokinase, or presence of a proteolytic contaminant.

Thrombin.—The mechanism by which fibrinogen is converted to the fibrin monomer through the action of thrombin with the liberation of peptide material has been described in some detail in two publications by Bailey & Bettelheim (144, 145). This proteolysis does not concern the C-terminal end of fibrinogen, since carboxypeptidase releases phenylalanine (together with small amounts of three unidentified amino acids) from both fibrinogen and fibrin; however, the N-terminal glutamic acid residue of fibrinogen is replaced by 2 or 3 glycine residues as has previously been described in a joint note from two laboratories (146). This glutamic acid appears in peptide form ("peptide A") in the nonprotein fraction released during the action of thrombin; a second "peptide B" which is released simultaneously contains a lysine residue but no N-terminal groups. Each peptide has a molecular weight of approximately 3,000, and an isoelectric point of 3.1 to 3.2. The amino acid composition of each peptide, determined qualitatively by column, paper, and partition chromatography is furnished. It should be noted that the composition of peptide B agrees with previous, more quantitative, data of Kominz & Laki (147); a difference exists in peptide A which Bettelheim describes, in contrast with the earlier workers, as being devoid of lysine and alanine. However, since the peptide material is stated to be exceptionally labile, the chromatographic methods employed may well have caused the partial degradation of peptide A during its isolation. Thrombin therefore appears to be a highly specific endopeptidase, and since it is known to hydrolyze tosyl.Arg.OMe [Sherry & Troll (148)], the possibility is advanced that an Arg.Gly linkage may be involved in the conversion of fibrinogen to fibrin. Inhibition of thrombin activity by DFP has been mentioned by Bailey & Bettelheim (144) and is described in greater detail by Gladner & Laki (149); this inhibition is partially overcome by hydroxylamine. The conversion of fibrinogen to fibrin by papain is insensitive to DFP.

Cathepsins.—Izumiya & Fruton (150) have continued the investigations of the Yale laboratory into the specificity of cathepsin C. Particular emphasis was placed on hydrolytic studies at pH 5 since at this pH nonenzymatic cyclization and replacement reactions are minimized. In general, esters and amides of the general type NH_2.CHR.CO.NH.CHR′.CO-X (R′ designates a phenylalanine or tyrosine residue) are split at the same rate. When R is a hydrogen atom methylation of the free amino group decreases, and acylation abolishes activity of the resulting compounds as substrates. Substitution of proline for the N-terminal amino acid results in easily hydrolyzable derivatives, which, moreover, do not appear to undergo appreciable replacement reactions or polymer formation at pH 7.5.

The digestion of thyroglobulin by an extract of beef thyroid, followed by the determination of the rate of liberation of trichloroacetic acid-soluble iodine, has been studied by Abelin & Bigler (151); the pH optimum of this hydrolysis is 4.5 to 4.8, close to the isoelectric point of the substrate. The use of cellulose anion exchangers with water-CO_2 systems in the purification

of proteins was illustrated by a fractionation of partially purified bovine kidney cathepsin (152).

Wattiaux & de Duve (153) have continued their investigations into the localization and release of intracellular cathepsins and have observed that addition of the detergent Triton X-100 with the substrate is more efficient and convenient than the usual methods for liberation of enzymatic activity from granular material ("lysosomes"). Variation in detergent concentration has an almost "all or none" effect, within narrow limits, on the liberation of enzyme.

Inactivation of insulin.—The interesting finding of materials in rat liver extracts which inactivate insulin, reviewed previously (154), has been extended in the laboratories of Williams and of Mirsky. This reaction is usually followed by release of trichloroacetic acid-soluble I^{131} from labeled insulin, and in the reviewers' opinion caution should be used in the extrapolation of data so obtained to unlabeled insulin. For this approach to be valid, the addition of unlabeled insulin to the reaction mixture should be expected to produce a decrease in the rate but not in the extent of release of I^{131}. While the effect on rate appears to be found consistently, the amount of total I^{131} released is decreased in some of the experiments reported (155, 156), but not in others (157). The enzymatic activity is contained in the supernatant fraction of a rat liver homogenate centrifuged at 110,000 g in 0.25 *M* sucrose (157). The name "insulinase" (158) probably implies too high a specificity, inasmuch as α-corticotropin, α-casein, glucagon, and (to a slight extent) growth hormone depress the rate of release of I^{131} *in vitro* (156), and α-corticotropin, α-casein, and insulin also potentiate the glycogenolytic action of glucagon (159); these proteins may therefore all be regarded tentatively as competing substrates or inhibitors. Serum albumins, α-lactalbumin, and ribonuclease are without effect in either test system. Inhibition of the activity *in vitro* by relatively high concentrations of some amino acids (160) and *in vivo* by a presumably protein-free preparation from beef liver (161) has been reported.

The cathepsin activity of Walker carcinoma is of the same order as that of normal tissue in rats [Babson, (162)]. This appears to have been the first study of this kind since that of Maver & Dunn (163) which also showed fairly, but not abnormally, high activities in various neoplasms. On the other hand, noncancerous maturing cells, such as reticulocytes (164) or regenerating larval tissue following amputation (165), show an increase in cathepsin activity over normal controls.

Exo- and endopeptidases of unicellular organisms.—Félix & Labouesse-Mercouroff (166) report a twentyfold enrichment of a carboxypeptidase from brewer's yeast which is stable between pH 4.7 and 7 and is optimally active at pH 6. In addition to peptides hydrolyzed by pancreatic carboxypeptidase (e.g., cbzo.Gly.Leu) it also attacks cbzo.Gly.Gly and DNP.Gly.Gly. Its inhibition by EDTA is readily reversed by dialysis. The proteinase (167) and peptidase (168) activities derived from broken cell preparations of

Lactobacillus casei have been investigated by Brandsaeter & Nelson. A survey of 542 strains of *Aspergillus* as to their proteolytic activity has indicated the occurrence of two classes of endopeptidases, with general characteristics of pepsin and of trypsin or papain, respectively (169). A preliminary report by Ogle & Logan (170) concerns the partial purification of a gelatin- and collagen-splitting enzyme from *Clostridium histolyticum* which liberates free amino groups derived from glycine moieties of the substrate.

The characteristics of two proteinases occurring in autolysates of plasmolyzed yeast have been described by Lenney (171). The two enzyme activities can be differentiated by the effect of pH on their thermal stability, and also by the effect of urea; "Proteinase A" (substrate: acid-denatured hemoglobin; pH optimum approx. 3.7) is destroyed by 5.5 *M* urea at pH 2 to 4, while "Proteinase B" (substrate: urea-denatured hemoglobin; pH optimum 6.2) shows a rather remarkable net activation by urea within a narrow range of pH around 5 (the activating effect is overshadowed by denaturation at higher and lower pH values). While in many respects Proteinase B resembles papain, it is not activated by cyanide or cysteine (though cyanide reverses the inhibition by *p*-chloromercuribenzoate), and iodoacetate is without effect on its activity.

Viswanatha & Liener (172) have isolated a proteinase as a crystalline mercuri-derivative from the supernatant of cultures of *Tetrahymena pyriformis W* (*T. geleii*). This enzyme, which is devoid of tyrosine, also resembles papain when tested with hemoglobin as substrate. However, it has no effect on bz.Arg.ONH_2 at pH 6.4 to 7 while readily attacking bz.Arg.OMe. Of various other synthetic peptides investigated, only cbz.Glu.Tyr serves as a substrate at pH 4.0 though not at pH 6.5. The kinetics of this hydrolysis appear to be complicated because of strong product inhibition.

Natural inhibitors of endopeptidases.—Ambe & Sohonie (173) report further experiments on the crystalline trypsin inhibitor obtained from Indian field beans. The inhibitor level decreases with age in the plant but increases in the seeds. This finding is related to a decrease in protein synthesis in the plant and an accumulation of proteins in the seeds. The same authors (174) have also obtained two crystalline, thermolabile trypsin inhibitors from potatoes, one of which is soluble in 2.5 per cent trichloroacetic acid, and the other extractable from the residue with 1 per cent NaCl. Bier *et al.* (175) have determined the dissociation constants of the complexes formed between trypsin inhibitor from human serum and several acylated trypsins.

The inhibition of several endopeptidases by ox lung tissue was reported previously by Astrup (176); a partially purified inhibitor preparation from this source has now been obtained (177). The inhibitor is absent from lung tissue of most other species, including man. De Barbieri & Scevola (178) describe the combination of trypsin with an inhibitor derived from human placenta.

The inhibitory action of allicin ($CH_2{:}CH{\cdot}CH_2{\cdot}SO{\cdot}S{\cdot}CH_2{\cdot}CH{:}CH_2$)

on several enzymes including papain has been investigated by Wills (179). The compound was found to be reasonably specific for enzymes dependent on sulfhydryl groups. The inhibition could be partially reversed by prompt addition of sulfhydryl compounds but became irreversible after 15 minutes.

Substrate Specificity of Endopeptidases: Studies with Protein Substrates

Early studies of the sequence of amino acids in partial breakdown products of proteins involved the more or less haphazard liberation of small peptide chains by partial acid hydrolysis. More recently, this has been supplemented by the more specific hydrolysis of peptide bonds by means of purified proteolytic enzymes. As a by-product of this work, it is becoming possible to arrive at answers as to whether endopeptidases do indeed exhibit the same structural requirements in proteolysis as those established with the use of model structures.

Chymotrypsin.—Agreement of specificity determination by means of synthetic substrates with results obtained by protein hydrolysis is quite uniform. Acher, Laurila & Fromageot (180), in a study of the proteolysis of heat-denatured lysozyme, show that chymotrypsin usually cleaves, as predicted, at the carboxyl side of Tyr, Phe, or Met, though the peptide Glu.Ser.Phe.Asp was found to be resistant to chymotryptic attack. According to Harris & Roos (181), the linkage Met.Glu is also unaffected by chymotrypsin; these two results may indicate an inhibitory effect of a dicarboxylic acid, when it constitutes the NH-contributing moiety of a normally sensitive peptide linkage. Hirs, Stein & Moore (182), in their excellent and systematic studies on the amino acid sequence of ribonuclease after performic acid oxidation, find chymotryptic hydrolysis to take place also at the carboxyl side of Leu, Asp(NH_2), and, in at least one case, of His.

Trypsin.—Trypsin yields results which agree with its predicted specificity. Thus, Hirs, Moore & Stein (183) have isolated 13 peptides in 50 to 100 per cent yield after tryptic digestion of ribonuclease, whereas 14 to 15 were expected on the basis of the presence of ten lysine and four arginine residues. Bromer *et al.* (184) find the linkage Try.Leu to be one of the major sites of splitting of glucagon by trypsin, in addition to Lys.Tyr and Arg.Ala; however, it remains to be established if this finding may not be due to the known chymotryptic activity of trypsin (111) and the demonstrated hydrolysis of this bond by chymotrypsin. Thaureaux & Acher (185) have likewise found consistent results in their studies of the tryptic hydrolysis of lysozyme. They ascribed the appearance of free arginine after tryptic digestion to an Arg.Arg or Arg.Lys sequence, and that of leucine [which is C-terminal (186)] to a C-terminal Lys.Leu or Arg.Leu. A C-terminal dicarboxylic acid adjacent to a dibasic one appears to inhibit the action of trypsin. Thus, Harris & Roos (181) find C-terminal Lys.Asp to be stable to tryptic hydrolysis, in accord with earlier results by Plentl & Page (187) with C-

terminal Lys.Glu. Internal Lys.Asp (182) and Lys.Glu (187) bonds are however cleaved by trypsin.

Pepsin.—In contrast with chymotrypsin and trypsin, specificity of pepsin is far lower than was originally thought. Acher *et al.* (180) find the linkages Arg.Gly, Ala.Tyr, and Phe.Glu, as well as the amino side of alanine residues to be susceptible to peptic attack. Cole & co-workers (188), in their study of the C-terminal end of α-corticotropin, have obtained a variety of leucine-containing peptides by peptic hydrolysis. Inasmuch as this hormone had previously been found to contain only one leucine moiety which is located near the C-terminal end (189), it is concluded that in the more extended C-terminal sequence here established, which is Asp.Glu.Ala.Ser. Glu.Ala.Phe.Pro.Leu.Glu.Phe, pepsin is able to hydrolyze all linkages except perhaps that of Pro.Leu.

Subtilisin.—This enzyme appears to be highly specific in the conversion of ovalbumin to plakalbumin, recently reviewed by Desnuelle & Rovery (190). This specificity may well be due to a protection of otherwise susceptible linkages in native ovalbumin, since subtilisin hydrolyzes other proteins at a great profusion of peptide linkages. Thus, 23 out of 49 peptide bonds in crystalline pork insulin, including all but one of those involving the carboxyl side of leucine residues are cleaved [Meedom (191)]; in confirmation, Haugaard & Haugaard (192) report the liberation of peptides with an average chain length of three to four amino acids from insulin with carboxyl groups of eight and amino groups of eleven different amino acids. Richards (193) concludes that about 25 per cent of all peptide bonds are split during the action of subtilisin on ribonuclease. Bromer *et al.* (184), in the previously-mentioned study of the amino acid sequence of glucagon, find subtilisin to hydrolyze the following ten linkages out of a total of 28: Glu(NH_2).Gly, Phe.Thr*, Ser.Asp, Ser.Lys, Tyr.Leu*, Arg.Arg (a rather interesting hydrolysis), Glu(NH_2).Asp, Phe.Val*, Try.Leu*, and Met.Asp (NH_2). It is interesting to note that those peptide linkages which are marked with an asterisk are hydrolyzed by chymotrypsin also; a Tyr.Ser bond which is broken by chymotrypsin is not attacked by subtilisin.

What appears to be a novel method of following the stepwise enzymatic degradation of lysozyme by chymotrypsin, trypsin, papain (at pH 5.0, 7.5 and 8.5), and pepsin (at pH 4) has been employed by Hurst & Fox (194). The principal features of the method have previously been used by DeFontaine & Fox (195) in the study of much smaller peptides. In the present study it is shown that on prolonged incubation (up to 240 hr.) far more peptide linkages are split than would be expected on the basis of previously determined specificities (in the case of those enzymes where evidence from other sources is available). However, in the opinion of the reviewers, the experimental data do not indicate the exclusion of transpeptidation and/or bacterial contamination, and shorter digestion periods (24 hr. was the shortest interval examined) appear to yield specificities essentially in agreement with the experience of other laboratories.

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METABOLISM OF LIPIDES[1,2]

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Since it is impossible to cover the entire field of lipide metabolism within reasonable limitations of space, this review will be confined to selected topics, for the most part not reviewed last year.

METABOLISM OF FATTY ACIDS

Enzymatic Oxidation of Fatty Acids

Advances in this field since the masterly review by Lynen in 1955 (1) have largely been confined to working out the details of the separate enzymatic steps of the fatty acid oxidation cycle. The rapid and spectacular elucidation of the pattern of β-oxidation in animal tissues must be regarded as one of the outstanding achievements of modern biochemistry.

A uniform system for naming the enzymes of the fatty acid oxidation cycle has been set forth by representatives of several laboratories active in this field (2, 3). Such a uniform nomenclature would be very desirable, since many of these enzymes have been assigned different names by various workers who have studied them. Thus the enzyme named "palmityl CoA dehydrogenase" by Beinert *et al.* (4) should properly be called "lauryl dehydrogenase" since the words "CoA" should not appear in the name of such an enzyme (2, 3) and the enzyme exhibits maximum activity with lauryl CoA as substrate.

Fatty acyl dehydrogenases.—Green and his collaborators (4 to 7) have reported details of their studies on the dehydrogenation of fatty acyl derivatives of CoA, which proceeds according to the equation:

$$RCH_2CH_2CO{-}S{-}CoA \rightleftharpoons RCH{=}CHCO{-}S{-}CoA + 2H \qquad 1.$$

Three separate enzymes, with different specificities for CoA thioesters of fatty acids of varying chain length, are distinguished by these workers. One

[1] The survey of the literature on the topics covered in this review was completed in November, 1956.

[2] The following abbreviations are used in this chapter: AMP, ADP, and ATP for adenosinemonophosphate, adenosinediphosphate, and adenosinetriphosphate; CDP-choline for cytidine diphosphate choline; CDP-ethanolamine for cytidine diphosphate ethanolamine; CoA for coenzyme A; CTP for cytidine triphosphate; DPN and DPNH for oxidized and reduced diphosphorpyridine nucleotide; ETF for electron-transferring flavoprotein; FAD for flavin adenine dinucleotide; GDP and GTP for guanosine diphosphate and guanosine triphosphate; ITP for inosine triphosphate; P_i for orthophosphate; P—P for pyrophosphate; TPN, TPNH for oxidized and reduced triphosphopyridine nucleotide; and UTP for uridine triphosphate.

[3] The author is indebted to Sylvia Wagner Smith, Louise Borkenhagen, and Michael Sribney for aid in searching the literature, and to Drs. H. G. Williams-Ashman and S. B. Weiss for helpful discussions.

of these enzymes (G) is a green cuproflavoprotein which attacks CoA esters of chain length C_4 to C_8; another (Y) is a yellow flavoprotein specific for chain lengths C_4 to C_{16}; a third enzyme (Y′, more recently renamed "palmityl CoA dehydrogenase") is active toward chain lengths of C_6 to C_{16} or higher.

Y has been purified from pig liver (5) and freed of Y′ and G. The molecular weight is about 200,000. It has an absorption spectrum characteristic of a flavoprotein, but some anomalies are reported. The enzyme may apparently contain either one or two moles of flavin (identified as FAD) per mole of protein, depending upon the methods of preparation. It contains one atom of iron per six moles of flavin, and negligible amounts of copper.

The preparation of Y′ (palmityl CoA dehydrogenase) from acetone powders of pig heart has been described by Hauge, Crane & Beinert (4). Like Y, this enzyme contains FAD as the prosthetic group, but has no significant amounts of iron. The highest activity is observed with lauryl CoA as substrate, but activity with palmityl CoA is 70 per cent of maximal. The enzyme is completely inactive towards butyryl CoA.

Y, Y′, and G may be easily reduced by their appropriate substrates, but can be reoxidized by most electron acceptors only in the presence of a specific flavoprotein called the electron-transferring flavoprotein (ETF). ETF has been purified about fiftyfold from extracts of acetone powders of mitochondria from pig liver, by procedures which include fractionation as the Zn complex (6). The molecular weight is in the range 30,000 to 70,000, and the prosthetic group appears to be FAD. Only traces of iron or copper are found in the enzyme.

ETF exhibits variable activity toward cytochrome-*c* as electron acceptor, but great increases are noted after treatment with acid ammonium sulfate. It is suggested that this treatment unmasks the active site on the enzyme which reacts with cytochrome-*c*.

Hauge (7) has studied the interaction of ETF and the fatty acyl dehydrogenases, and also reports a value of -0.017 v. for the oxidation-reduction potential, E_0', of the system butyryl CoA-crotonyl CoA.

Enoyl CoA hydrase.—Crotonase (enoyl CoA hydrase) has been isolated in crystalline form from ox liver by Stern, Del Campillo & Raw (8) and its properties studied in detail (9). The decrease in optical density at 263 mμ as crotonyl CoA is hydrated offers a convenient assay for the enzyme.

Both *cis* and *trans* double bonds in CoA derivatives of chain length C_4 to C_9 are hydrated by the enzyme, with rates decreasing with increasing chain lengths. Crotonyl-S-pantetheine reacts at only 0.013 per cent of the rate of crotonyl CoA; nevertheless, Stern (10) has suggested that the metabolism of fatty acids as thioesters of pantetheine rather than CoA may constitute an alternative metabolic pathway. Double bonds in the 3–4 position are attacked as well as those in the 2–3 position, enabling the enzyme to act as an isomerase, e.g. in the conversion of vinylacetyl CoA to crotonyl CoA. Double bonds in the 4–5 position are not attacked.

It was reported by Stern & Del Campillo (9), and previously by Stern, del Campillo & Lehninger (10a), that the product of the enzymatic hydration of both crotonyl CoA (*trans*) and isocrotonyl CoA (*cis*) is L-β-hydroxybutyryl CoA, as measured by direct optical test. These workers have also found a racemase in animal tissues, which catalyzes the reaction:

$$\text{D-}\beta\text{-hydroxybutyryl CoA} \rightleftharpoons \text{L-}\beta\text{-hydroxybutyryl CoA} \qquad 2.$$

Apparently DPN is not needed for this racemase reaction.

On the other hand, Wakil (11) has presented evidence that the enzymatic hydration of crotonyl CoA yields L-β-hydroxybutyryl CoA, while hydration of isocrotonyl CoA yields the D isomer. In these experiments, after hydration of crotonyl and isocrotonyl CoA with crotonase, the resultant products were hydrolyzed with mild alkali, and the configuration of the free β-hydroxybutyrate was determined, using an enzyme specific for D-β-hydroxybutyrate.

Wakil (12) has also found a β-hydroxybutyryl CoA dehydrogenase in beef liver which is specific for the D isomer, in contrast to the enzyme described by Lehninger & Greville (13) which attacks the L compound only; both enzymes are DPN linked. It is possible that the combined action of the two enzymes may constitute a racemase system, with acetoacetyl CoA as intermediate.

Acetoacetyl-succinic thiophorase and acetoacetyl thiolase.—Stern, Coon & del Campillo (14, 15) have studied the following reactions in extacts of pig heart:

$$\text{succinyl CoA} + \text{acetoacetate} \rightleftharpoons \text{acetoacetyl CoA} + \text{succinate} \qquad 3.$$

$$\text{acetoacetyl CoA} + \text{HS—CoA} \rightleftharpoons 2\ \text{acetyl CoA} \qquad 4.$$

The thiophorase catalyzing reaction 3 was purified more than one thousandfold and its properties determined (15). It is completely specific for succinate but will attack β-keto acids with 4 to 6 carbon atoms, including α-methyl derivatives (16). Pantetheine derivatives cannot be substituted for CoA esters.

The characteristic absorption spectrum of acetoacetyl CoA, with a band at 303 mμ, is of great aid in studying Reactions 3 and 4. Stern (17) has carried out a thorough investigation of the optical properties of acetoacetyl CoA and related compounds with particular attention to the effects of magnesium and other cations in forming chelates with the enolate ion forms of these compounds.

Oxidation of fatty acids in tumors.—Oxidation of fatty acids in cell-free preparations of tumor tissues has been described in a number of papers by Emmelot & Bos and their co-workers (18 to 21). Earlier attempts to demonstrate these reactions in cell-free preparations had been unsuccessful, although the work of Weinhouse (22) had made it clear that such a process must be taking place in the intact tumor cell.

Oxidation of fatty acids in plants.—The oxidation of fatty acids by three

separate intracellular fractions of peanut cotyledons has been studied by Stumpf and his collaborators and found to occur by strikingly different pathways. Humphreys *et al.* (23) found that microsomes from cotyledons of germinating peanuts oxidize palmitic acid, labeled either in the carboxyl group or internally (2, 3 or 11 positions) with the production of radioactive carbon dioxide. No cofactor requirement was detected for the oxidation of carboxyl-labeled palmitate, but DPN was necessary for the production of radioactive carbon dioxide from internally labeled palmitate. This reaction sequence is obviously different from the β-oxidation observed in animal tissues. In continuation of this work, Humphreys & Stumpf (24) prepared an extract of soluble enzymes from the microsomes by treatment with sodium choleate. After precipitation of the enzyme with ammonium sulfate, added DPN was necessary for production of radioactive CO_2 from carboxyl as well as internally labeled palmitate. A further, as yet unidentified, factor is present in boiled extracts of peanut cotyledons; this cofactor activates the enzyme to oxidize carboxyl-labeled palmitate in the absence of DPN and increases the rate of CO_2 production from internally labeled palmitate in the presence of DPN. The activator has properties suggestive of an organic acid, but apparently is not glycolic or lactic acid. Intermediates in the reactions catalyzed by the microsomal system have not yet been identified.

The soluble enzyme fraction, obtained after removal of mitochondria and microsomes by high speed centrifugation, contains a fatty-acid-oxidizing system quite different from the microsomal enzyme(s). Only the carboxyl group of palmitic acid appears as CO_2; internally labeled palmitate does not give rise to radioactive CO_2. After precipitation of the enzymes with ammonium sulfate, glycolic, lactic, or some other L-α-hydroxy aliphatic acid in substrate concentration is necessary for activity (25). However, α-ketopalmitic acid is decarboxylated in the absence of added glycolic acid, and at a higher rate than palmitate. These results suggest the following reaction sequence:

$$\text{palmitic acid} \xrightarrow[\text{acid}]{\text{glycolic}} \alpha\text{-ketopalmitic acid} \qquad 5.$$

$$\alpha\text{-ketopalmitic} \longrightarrow \text{pentadecylic aldehyde} + CO_2 \qquad 6.$$

The function of glycolic acid in Reaction 5 is apparently to undergo oxidation by glycolic oxidase, thus furnishing hydrogen peroxide for the oxidation of palmitic acid by a fatty acid peroxidase [Stumpf (26)].

Although the microsomes and supernatant solution from peanut cotyledons oxidize fatty acids by pathways very different from the processes thus far observed in animal tissues, the mitochondria from peanut cotyledons possess a fatty acid oxidase system essentially similar to that found in animal tissues [Stumpf & Barber (27)]. It is necessary to supplement the plant mitochondria with ATP, DPN, TPN, CoA, Mn, glutathione, and an intermediate of the Krebs cycle in order to obtain maximum activity. Apparently,

the acetyl CoA derived from fatty acid oxidation in mitochondria from peanut cotyledon also is oxidized via the Krebs cycle, just as in animal tissues. Some accumulation of acetoacetate in the plant system also occurs.

The importance of β-oxidation in higher plants, for the function of aryloxyalkylcarboxylic acids (herbicides), has been discussed by Fawcett *et al.* (28).

Oxidation of fatty acids in bacteria.—The reduction of vinyl acetate to butyrate in cell-free extracts of *Clostridium kluyveri* has been shown by Peel & Barker (29) to require catalytic amounts of "high energy acetate," i.e., acetyl CoA or acetyl phosphate. These results, in conjunction with previous work of Barker and his collaborators (30, 31, 32), indicate that in this microorganism, as in animal tissues, the active substrates in fatty acid oxidation and synthesis are thioesters of CoA. Evidence for a similar process of β-oxidation of fatty acids in other microorganisms has appeared in papers by Ivler, Wolfe & Rittenberg (33), Webley, Duff & Farmer (34), and Dagley (35).

Nonenzymatic decarboxylation of fatty acids.—The decarboxylation of labeled stearic and palmitic acids was found by Geyer *et al.* (36) to proceed with unexpected ease in a nonenzymatic reaction when the fatty acid was incubated in the presence of ascorbic acid at pH 7.1 and 37° under oxygen. Occurring as it does under approximately physiological conditions, this reaction may be a pitfall in the study of the enzymatic oxidation of fatty acids.

The Enzymatic Synthesis of Fatty Acids

Since reactions of the fatty acid oxidation cycle are reversible, it might be presumed that fatty acid synthesis is simply a reversal of the oxidative process. In the experiments of Stadtman & Barker (37, 38) the same cell-free extracts of *Clostridium kluyveri* which oxidized short chain fatty acids when incubated under an atmosphere of air or oxygen, synthesized fatty acids from acetyl phosphate plus acetate under an atmosphere of hydrogen, suggesting that in this microorganism the synthesis of fatty acids is simply a reversal of oxidation.

In mammalian tissues, the situation is probably much more complex. Fatty acid synthesis is a reductive, endergonic process rather than oxidative and exergonic, and it seems obvious that optimal conditions for synthesis and oxidation may well be very different. In addition, the nutritional status of the animal determines whether net synthesis or breakdown of fats takes place, and one may suspect that important hormonal controls may be involved as well. Furthermore, recent work has made it apparent that other control mechanisms, potentially of great physiological interest, such as the intracellular localization of enzyme systems and specific functions of pyridine nucleotides, may also be in operation.

Intracellular localization of fatty acid synthesis.—The complex enzyme system which catalyzes the oxidative degradation of fatty acids is localized in the mitochondria of the liver cell (39, 40). Reports from several labora-

tories indicate that particle-free soluble extracts of rat liver (41), pigeon liver (42, 43, 44), and mammary gland of the rat and the rabbit (45, 46, 47) catalyze the synthesis of fatty acids, when properly supplemented with cofactors. In contrast, it appears that fatty acid synthesis in isolated mitochondria has not yet been demonstrated, which suggests the possibility that synthesis and breakdown take place in different regions of the cell.

Functions of DPNH and TPNH.—The biosynthesis of fatty acids from acetate involves two reductive steps, the conversion of β-ketoacyl CoA esters to the β-hydroxy derivatives, and reduction of enoyl CoA esters to saturated compounds. It has been firmly established that the former reaction is DPN-linked, but the physiological reductant in the latter has remained in doubt (1). Langdon (41) has more recently presented evidence that both TPN and DPN are required for the synthesis of fatty acids in enzyme extracts from rat liver. He was further able to demonstrate spectrophotometrically the oxidation of TPNH (but not DPNH) by crotonyl CoA, indicating a specific function of TPNH in the reduction of unsaturated CoA esters. This conclusion was supported by Brady, Mamoon & Stadtman (44) who found that the stimulatory effect of citrate on fatty acid synthesis in soluble extracts of pigeon liver results, in part at least, from the generation of TPNH via isocitric dehydrogenase.

While DPNH is rapidly oxidized by molecular oxygen in isolated mitochondria, with coupled phosphorylation (48), recent work of Kaplan *et al.* (49) indicates that TPNH is only slowly oxidized, with little or no coupled phosphorylation. These workers suggest that TPNH may be specifically reoxidized during fatty acid synthesis, and that transhydrogenase (50) may be a potential regulator controlling fatty acid breakdown or synthesis.

Other properties of fatty-acid synthesizing systems.—Popjak & Tietz (45, 46) have studied the biosynthesis of fatty acids in a soluble enzyme system prepared by high speed centrifugation of an extract of rat mammary gland. About 25 per cent of the radioactivity of fatty acids synthesized by this system from labeled acetate was present as long-chain (C_{12} to C_{18}) fatty acids; the remainder consisted of short and intermediate chain acids. Maximal incorporation required the addition of ATP, α-ketoglutarate, and malonate. After treatment of the extract with Dowex-1 ion exchange resin to remove cofactors, CoA and DPN were also needed (46).

Fractionation of a soluble extract of rabbit mammary gland by Hele & Popjak (47) resulted in a considerable increase in fatty acid synthesis per mg. of protein. Upon addition of acetate to an enzyme system containing ATP, CoA, cysteine, $MgCl_2$, and DPNH, a rapid oxidation of DPNH was noted; fatty acid synthesis could thus be measured spectrophotometrically. In these experiments and those of Tietz & Popjak (46) it would appear that DPNH may act as reductant in the conversion of unsaturated CoA esters to the saturated compounds. However, purified fatty acyl dehydrogenases do not react with DPNH, according to Green *et al.* (51) and Seubert & Lynen (52). It is possible that extracts of mammary glands contain a DPN-linked

enzyme, lacking in liver tissue, which reduces fatty acyl dehydrogenases (which do not themselves react with DPNH).

At least three separate enzyme fractions and many cofactors are needed to reconstitute the fatty acid synthesis system after fractionation, according to Gibson & Jacob (43) and Wakil, Porter & Tietz (42). Amongst the cofactors needed are ATP, CoA, DPN, TPN, glutathione, lipoic acid, and isocitrate. Some progress has been achieved in localizing specific enzymatic steps in the subfractions (43).

The puzzling finding by Brady & Gurin (53) that acetyl CoA is a less efficient precursor of labeled fatty acids than free acetate in the presence of CoA and ATP has been explained by Brady, Mamoon & Stadtman (44): free CoA, released in relatively large amounts when acetyl CoA is added in substrate quantities, inhibits fatty acid synthesis and promotes fatty acid oxidation.

Effects of starvation.—It is well known that fatty acid synthesis by liver slices from fasted rats is depressed, as compared with slices from fed rats [cf. Lyon *et al.* (54)]. Similar effects of starvation have been found in adipose tissue (55, 56). In an extension of these observations to cell-free enzyme systems, Catravas (57) reported that a factor found in the liver of fed rats stimulates fatty acid synthesis in homogenates derived from livers of starved rats. The unidentified factor is said not to be glycogen, hexosediphosphate, or any of a number of known cofactors tested.

Lipide synthesis in yeast.—Differential centrifugation of cell-free extracts of *Saccharomyces cerevisiae* yields a "small particle fraction" and a soluble supernatant fraction, both of which are necessary for fatty acid synthesis [Klein & Booher (58, 59)]. These combined fractions also catalyzed the incorporation of labeled acetate into nonsaponifiable lipides.

Metabolism of Short-Chain and Branched Chain Fatty Acids

Enzymatic activation of acetate.—Berg (60, 61) has postulated the following reaction scheme to account for the formation of acetyl CoA from ATP, CoA, and acetate:

$$\text{adenosine—P—P—P} + \text{CH}_3\text{COOH} \rightleftharpoons \text{CH}_3\text{CO—P—adenosine} + \text{P—P} \qquad 7.$$

$$\text{CH}_3\text{CO—P—adenosine} + \text{CoA—SH} \rightleftharpoons \text{CH}_3\text{CO—S—CoA} + \text{adenosine—P} \qquad 8.$$

In support of this formulation, Berg found that equilibration of inorganic pyrophosphate and ATP was dependent upon the presence of acetate, in contrast to the earlier findings of Jones *et al.* (62) who worked with cruder enzyme preparations. Acetyl adenylate was synthesized by chemical means (63) and found to react with inorganic pyrophosphate to form ATP, and with CoA to form acetyl CoA, which are the properties required of an intermediate in Reactions 7 and 8. However, no accumulation of acetyl adenylate could be detected, nor did unlabeled acetyl adenylate act as an isotopic diluent for C^{14}-labeled ATP. Similar evidence for butyryl adenylate as an inter-

mediate in the enzymatic activation of butyrate has been reported by Talbert & Huennekens (64) and Lee Peng (65).

Independent investigations in several laboratories point to a basically similar mechanism for activation of the carboxyl groups of amino acids, with the formation of amino acyl adenylates as possible intermediates. DeMoss & Novelli (66, 67) showed that synthetic leucyl adenylate has properties suggestive of those of an intermediate in the amino-acid-dependent exchange of inorganic pyrophosphate with ATP, catalyzed by an enzyme from *Escherischia coli*. Similar reactions have been found in liver by Hoagland (68) and in yeast by Berg (69). In liver, both ATP and GTP (or GDP) are required for the incorporation of amino acids into proteins in cell-free preparations of liver [Keller & Zamecnik (70)]. Similarly, a synthetic compound with properties resembling those of "carbonyl adenylates" may be an intermediate in the enzymatic activation of carbon dioxide by ATP, according to Bachhawat, Woessner & Coon (71).

These diverse lines of evidence seem to indicate a common pattern for the activation of carboxyl groups, whether in amino acids, fatty acids, or carbonic acid. The activation of carbon dioxide by ATP in the carboxylation of propionyl CoA to form methylmalonyl CoA appears, however, to proceed by an entirely different pathway (72), as does the direct phosphorylation of acetate by enzymes present in microorganisms (73).

Conversion of propionate to succinate.—The major pathway for the utilization of propionate in animal tissues involves its conversion to succinate, according to reports by Flavin *et al.* (72, 74), Katz & Chaikoff (75), and Lardy and co-workers (76, 77). Methylmalonate was discovered to be an intermediate in this reaction sequence by Flavin, Ortiz & Ochoa (74) and independently by Katz & Chaikoff (75). The following reactions have been described as occurring in pig heart extracts (74).

$$\text{propionate} + \text{ATP} + \text{CoA—SH} \rightleftharpoons \text{propionyl CoA} + \text{AMP} + \text{P—P} \qquad 9.$$

$$\text{propionyl CoA} + \text{CO}_2 + \text{ATP} \rightleftharpoons \text{methylmalonyl CoA} + \text{ADP} + \text{P}_i \qquad 10.$$

$$\text{methylmalonyl CoA} \rightleftharpoons \text{succinyl CoA} \qquad 11.$$

The succinyl CoA formed in Reaction 11 may then be converted to succinate by a variety of known enzymatic reactions, including transfer of the CoA moiety to acetoacetic acid (15), hydrolysis (78), or reaction with inorganic orthophosphate and GDP to form GTP (79).

The enzyme system catalyzing Reaction 10 has been purified about tenfold (72) and freed of the propionate-activating enzyme (Reaction 9). During the activation of carbon dioxide by ATP in this system, orthosphosphate is produced, and not pyrophosphate. Further, if fluoride, carbon dioxide, and ATP are incubated in the presence of the enzyme, considerable amounts of fluorophosphate are formed. A hypothetical reaction scheme to account for the over-all transformation in Reaction 10 on the basis of these results might be the following:

$$ATP + CO_2 \rightleftharpoons \text{carbonyl phosphate} + ADP \quad 10a.$$

$$\text{carbonyl phosphate} + \text{propionyl CoA} \rightleftharpoons \text{methylmalonyl CoA} + P_i \quad 10b.$$

Alternatively, in the presence of fluoride:

$$\text{carbonyl phosphate} + \text{fluoride} \rightleftharpoons \text{fluorophosphate} + CO_2 \quad 12.$$

Results consistent with the reaction sequence shown in Reactions 9 to 11 were obtained by Lardy & Adler (76) in a study of aged extracts of rat liver mitochondria and by Pennington & Sutherland (80) who also used rumen epithelium of the sheep; however, these workers did not detect methylmalonyl CoA as an intermediate. The carboxylation of propionate is much diminished in mitochondria from the livers of biotin-deficient animals (76); activity can be restored by the injection of biotin two or three days before sacrifice. A similar effect of biotin nutrition on the metabolism of five-carbon branched chain acids was noted by Fischer (81).

It is noteworthy that both methylmalonic (82) and dimethylsuccinic (83) acids are found in the urine of normal rats.

Degradation of isovaleryl CoA.—Bachhawat, Robinson & Coon (84) have presented further evidence in support of the following reaction scheme for the enzymatic degradation of isovaleryl CoA in extracts of rat liver and pig heart:

$$(CH_3)_2CHCH_2CO{-}S{-}CoA \rightleftharpoons (CH_3)_2C{=}CHCO{-}S{-}CoA + 2H \quad 13.$$

$$(CH_3)_2C{=}CHCO{-}S{-}CoA + H_2O \rightleftharpoons (CH_3)_2C(OH)CH_2CO{-}S{-}CoA \quad 14.$$

$$(CH_3)_2C(OH)CH_2CO{-}S{-}CoA + CO_2 \xrightarrow{ATP} (HOOCCH_2)(CH_3)C(OH)CH_2CO{-}S{-}CoA \quad 15.$$

$$(HOOCCH_2)(CH_3)C(OH)CH_2CO{-}S{-}CoA \rightarrow CH_3COCH_2COOH + CH_3CO{-}S{-}CoA \quad 16.$$

The oxidation of isovaleryl CoA in Reaction 13 is catalyzed by rat liver extracts with triphenyltetrazolium as electron acceptor. Since crotonase, which catalyzes Reaction 14, is also present in these extracts the actual product found is β-hydroxyisovaleryl CoA (determined as the hydroxamic acid derivative). The cleavage enzyme (catalyzing Reaction 16) may be inhibited by the addition of malate; under these conditions, β-hydroxy-β-methylglutaryl CoA accumulates as a product of Reaction 15. In a more detailed study of this carboxylation step, Bachhawat, Woessner & Coon (71) have reported that the over-all reaction may be broken down into two steps, each catalyzed by a separate enzyme:

$$CO_2 + ATP \rightleftharpoons \text{"Active } CO_2\text{"} + P{-}P \quad 15a.$$

$$\text{"Active } CO_2\text{"} + (CH_3)_2C(OH)CH_2CO{-}S{-}CoA \rightleftharpoons (HOOCCH_2)(CH_3)C(OH)CH_2CO{-}S{-}CoA \quad 15b.$$

A synthetic compound prepared by a reaction between ethylchloroformate and the silver salt of adenylic acid, and believed to be adenyl carbonate, is stated to carboxylate β-hydroxyisovaleryl CoA in Reaction 15b in the pres-

ence of a carboxylase enzyme which has been freed of the CO_2 activating system (Reaction 15a). The activation of carbon dioxide in this system would thus be strikingly similar to the activation of acetate, for which Berg (60) has proposed adenyl acetate as an intermediate, but very different from the ATP-linked activation of carbon dioxide studied by Flavin *et al.* (72).

Acetoacetate and acetyl CoA are formed upon the enzymatic cleavage of β-hydroxymethylglutaryl CoA in Reaction 16 [Bachhawat, Robinson & Coon (85)]. Apparently this step is not readily reversible.

Degradation of α-methylbutyryl CoA.—A scheme for the enzymatic degradation of the carbon skeleton of isoleucine which involves α-methylbutyryl CoA, tiglyl CoA, and α-methylacetoacetyl CoA has been put forward by Robinson, Bachhawat & Coon (16):

$$CH_3CH_2CH(CH_3)CO{-}S{-}CoA \rightleftharpoons CH_3CH{=}C(CH_3)CO{-}S{-}CoA + 2H \quad 17.$$

$$CH_3CH{=}C(CH_3)CO{-}S{-}CoA + H_2O \rightleftharpoons CH_3CH(OH)CH(CH_3)CO{-}S{-}CoA \quad 18.$$

$$CH_3CH(OH)CH(CH_3)CO{-}S{-}CoA + DPN \rightleftharpoons CH_3COCH(CH_3)CO{-}S{-}CoA + DPNH \quad 19.$$

$$CH_3COCH(CH_3)CO{-}S{-}CoA + CoA{-}SH \rightleftharpoons CH_3CH_2CO{-}S{-}CoA + CH_3CO{-}S{-}CoA \quad 20.$$

The oxidation of α-methylbutyryl CoA in Reaction 17 was followed by a dye-reduction method essentially similar to that used in the study of Reaction 13. Reaction 18, like Reaction 14, is catalyzed by crotonase. The production of acetyl CoA from tiglyl CoA (Reaction 19 plus 20) was shown to require DPN, indicating the participation of DPN in Reaction 19. It was also found that the cleavage of α-methylacetoacetyl CoA led to the formation of one mole of propionyl CoA (measured as the hydroxamic derivative) per mole of acetyl CoA.

Biosynthesis of branched chain acids.—Studies of the enzymatic cleavage of β-hydroxy-β-methylglutaryl CoA (Reaction 16) indicate that this step is not readily reversible (85). Since Rabinowitz (86) and Rudney (87) have found that β-hydroxyisovaleric and β-methylcrotonic acids may be synthesized from labeled acetate in homogenates of rat liver, it would appear that Reaction 16 may not be involved in the conversion of acetate to these branched chain compounds, which are regarded as closely related to active intermediates in the biosynthesis of cholesterol. An enzyme which catalyzes the condensation of acetyl CoA plus acetoacetyl CoA (rather than acetyl CoA plus free acetoacetate) has been found by Rudney (88). It is not known whether the product is a mono or di-CoA derivative of β-hydroxy-β-methylglutaric acid. This enzyme is apparently quite different from the cleavage enzyme described by Bachhawat *et al.* (85) and it may function as a bypass to Reaction 16 in the biosynthesis of branched chain fatty acids from acetate.

Branched chain acids as precursors of cholesterol.—There is considerable evidence that β-hydroxy-β-methylglutaryl CoA and β-methylcrotonyl CoA are closely related to some unidentified isoprenoid unit which is converted to cholesterol by mammalian liver [cf. Friedman *et al.* (89)]. Squalene or some

derivative of squalene is thought to be an intermediate in this process (89). Gurin and his collaborators (90) measured the conversion of labeled β-hydroxy-β-methylglutarate to squalene in cell-free extracts of rat liver, and found that the addition of unlabeled farnesol or farnesinic acid greatly diminished the incorporation of radioactivity into squalene. The recovered farnesinic acid was radioactive, suggesting that the observed effect was one of isotope dilution.

A new acetate-replacing factor for *Lactobacillus acidophilus* (ATCC 4963) was isolated from distillers' dried solubles by Wright *et al.* (91) and identified by Wolf and co-workers (92) as β-hydroxy-β-methyl-δ-valerolactone. The name divalonic acid suggested for this compound was later changed to mevalonic acid.

Mevalonic acid is converted extremely efficiently to cholesterol by cell-free liver preparations [Tavormina, Gibbs & Huff (94)]. As much as 43.4 per cent of the isotope from DL-mevalonic acid-2-C^{14} was recovered as cholesterol after incubation with homogenates of rat liver. On the assumption that only one optical isomer is active, this corresponds to a conversion of 86.8 per cent of the active form to cholesterol, a far greater conversion that has ever been observed with other five- or six-carbon branched chain acids. On the contrary, when carboxyl-labeled mevalonic acid was used, labeling of cholesterol was negligible, and the radioactivity was recovered principally as carbon dioxide (93), indicating that it is the carboxyl group which is lost during conversion to some presumed isoprenoid-like structure.

Metabolism of unesterified fatty acids of plasma.—The unesterified fatty acids of plasma are a quantitatively small (2 to 5 per cent) but metabolically important fraction of the total fatty acids of the blood, present not as free acids, but bound to plasma albumin (95, 96).

Injection of heparin into human subjects (97), rats (98), or dogs (99) leads to a marked increase in plasma unesterified fatty acids, suggesting that the presence of these acids is a reflection of the action of clearing factor, which has been described by Korn (100, 101) as a heparin-activated lipoprotein lipase, found in tissues as well as in the blood. Korn (102) has also shown that preincubation of lipoprotein lipase from chicken adipose tissue with "heparinase" (103) leads to inactivation, suggesting that heparin is a co-factor for lipoprotein lipase; this conclusion is also supported by the work of Robinson (104) and Engelberg (105). Clearing factor from postheparin plasma has been purified by Nikkilä & Pesola (106) and Hollet & Meng (107).

Independent investigations by Gordon & Cherkes (108), Dole (109), and Laurell (110) indicate that the levels of plasma unesterified fatty acid in man may be controlled by important hormonal mechanisms which are also involved in regulation of blood glucose. In these studies, it was found that prolonged fasting leads to an increase in plasma unesterified fatty acids, but ingestion of glucose or of a mixed meal brings about a prompt decrease.

Gordon & Cherkes (108) measured arteriovenous differences in the con-

centration of unesterified fatty acids, and concluded that 70 per cent of the energy requirement of the myocardial tissue in fasting subjects is met by absorption and combustion of these acids. The absorption of fatty acid is largely eliminated by the administration of glucose, indicating that glucose is the preferred substrate.

In a preliminary communication, Laurell (110) has reported that the level of unesterified fatty acid is high in the human subject with diabetic hyperglycemia, but falls upon the administration of insulin. This result points to an interesting difference between normal and diabetic subjects, since it would appear that the high blood glucose in the diabetic is not effective in depressing the levels of unesterified fatty acids.

Several workers (99, 108, 109, 110) have suggested, on the basis of these recent developments, that unesterified fatty acids may be the principal form in which fat is transported from depots to peripheral tissues for utilization. Some implications of recent work on this topic have been discussed by Anfinsen (111).

METABOLISM OF PHOSPHOLIPIDES

Biosynthesis of Phospholipides

Until the past few years, little progress had been made in working out the detailed chemical steps by which phospholipides are built up from simple precursors in the living cell. Although experiments with isotopic tracers in intact animals greatly enlarged our store of information about nearly every aspect of phospholipide metabolism, they could provide little information about the intermediate stages in biosynthesis. Numerous attempts were made to implicate various phosphorylated compounds as precursors of phospholipides, based on the relationships which should exist between the specific activities of a labeled product and its biological precursor after administration of a single dose of labeled precursor [Zilversmit, Entenman & Fishler (112)]. These efforts were unsuccessful, largely because of uncertainty as to whether the assumptions required for the theoretical treatment of Zilversmit *et al.* (112) are in fact justifiable in the living animal, and because novel and unexpected precursors could not be detected by this approach.

In isotopic tracer studies with tissue slices, Fries *et al.* (113) made the important discovery that an incorporation of P^{32} into phospholipides does not take place unless energy is supplied via coupled reactions. However, little further could be learned from tissue slice experiments, which are subject to many of the same limitations as experiments with intact animals.

Although early attempts (114) to demonstrate the synthesis of phospholipides in cell-free systems were unsuccessful, Friedkin & Lehninger (115) in 1948 showed that particulate preparations of rat liver incorporate P^{32} into phospholipides, provided that oxidative phosphorylation is maintained as a source of metabolic energy. These experiments confirmed the earlier observations of Fries *et al.* (113) as to the importance of an energy source, and

opened the way to a detailed study of the enzymatic steps leading to the biosynthesis of phospholipides.

Recent work in several laboratories on the enzymatic synthesis of phospholipides and triglycerides has led to the development of a rather complete picture of these processes, shown in simplified form in Figure 1. Phosphatidyl ethanolamine is synthesized by a pattern of reactions analogous

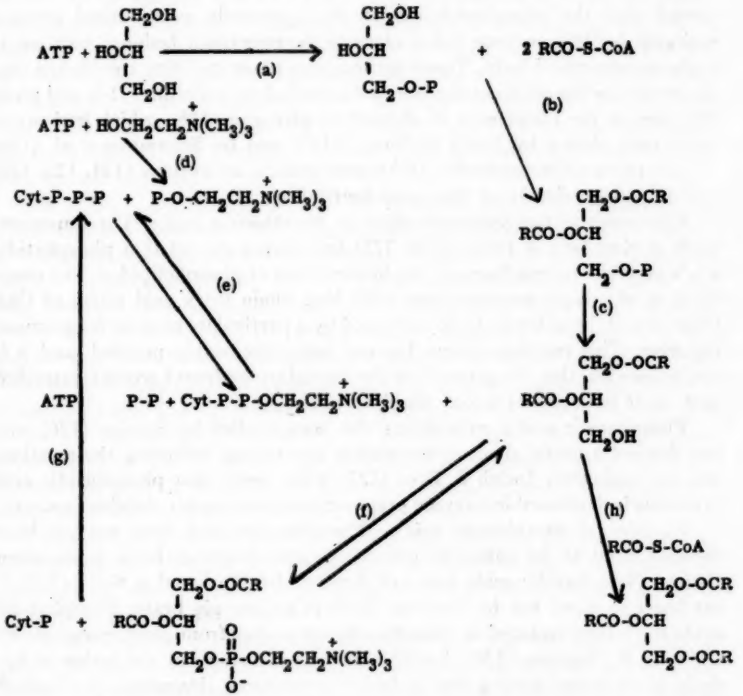


FIG. 1. Pathways for the enzymatic synthesis of phospholipides and triglycerides.

to those shown for phosphatidyl choline in Figure 1, but involving CDP-ethanolamine rather than CDP-choline. All of the reactions in Figure 1 have now been demonstrated in cell-free enzyme preparations with well-characterized intermediates, and each step has been shown to lead to net synthesis of product, eliminating the possibility that the reactions reflect merely isotope exchange rather than true synthesis.

L-α-*glycerophosphate as an intermediate in the biosynthesis of phospholipides.*—In a study of the incorporation of P_i^{32} into the phospholipide of a particulate enzyme preparation from rat liver, Kennedy (116) showed that

α-glycerophosphate is an obligate intermediate in the process. It was also found that the incorporation of isotope into a lipide fraction having the properties of a phosphatidic acid requires the preliminary conversion of P_i^{32} to ATP, and that the addition of free glycerol approximately doubles the rate of incorporation, suggesting that Reaction (*a*) (Fig. 1) is an essential step. Glycerokinase, the enzyme catalyzing the phosphorylation of glycerol in Reaction (*a*) was partially purified by Bublitz & Kennedy (117) who reported that the phosphorylation of the apparently symmetrical glycerol molecule by this enzyme takes place in asymmetrical fashion, yielding L-α-glycerophosphate only. These authors also presented direct evidence that glycerokinase functions in the biosynthesis of phospholipides (117) and probably also in the conversion of glycerol to glycogen (118), which had previously been shown by Swick & Nakao (119) and by Schambye *et al.* (120) to take place asymmetrically. D'Abramo and his co-workers (121, 122, 123) arrived independently at the same conclusions.

Conversion of L-α-*glycerophosphate to phosphatidic acid.*—The important work of Kornberg & Pricer (124, 125) first demonstrated that phosphatidic acids may be intermediates in the biosynthesis of phospholipides. The esterification of L-α-glycerophosphate with long chain fatty acid esters of CoA [Reaction (*b*)] was found to be catalyzed by a particulate enzyme from guinea pig liver. This enzyme system has not been extensively purified, and it is not known whether the primary or the secondary hydroxyl group is esterified first, or if both are attacked simultaneously.

Phosphatidic acid synthesis has also been studied by Stanley (126) who has devised a useful spectrophotometric method for following the reaction, and by Gallagher, Judah & Rees (127) who report that phosphatidic acid synthesis is depressed in enzyme preparations from copper-deficient animals.

Function of phosphatidic acids.—Phosphatidic acids have not yet been demonstrated to be naturally occurring components of fresh mammalian tissues. Phosphatidic acids were not detected by Marinetti & Stotz (128) in rat heart or liver, nor by Dawson (129) in guinea pig brain. Phosphatidic acids have been isolated in considerable quantities from plant materials by Chibnall & Channon (130), but this may be the result of the action of hydrolytic enzymes during the isolation procedures [Hanahan & Chaikoff (131)].

In view of the failure to find phosphatidic acids in any fresh tissues, the significance of the reactions leading to the synthesis of phosphatidic acids [Fig. 1, Reactions (*a*) and (*b*)] remained in doubt for some time, even though mounting evidence from several laboratories indicated a rapid synthesis of phosphatidic acid *in vitro* in whole-cell suspensions of guinea pig brain (129) and in slices of brain cortex and of pancreas (132), as well as in the cell-free systems studied by Kennedy (116) and Kornberg & Pricer (125). However, the statement by Marinetti & Stotz (128) that their failure to find phosphatidic acids in tissues leads to the conclusion that separate metabolic pathways are involved in phospholipide synthesis *in vivo* and *in vitro* must be

questioned. In the experiments described by these workers, the phospholipides extracted from rat heart or liver 18 hr. after the administration of P_i^{32} to the intact animal were chromatographed on paper impregnated with silicic acid. They were unable to detect significant radioactivity in the zones in which phosphatidic acids were expected to appear. In such an experiment, the amount of radioactivity detected in a given fraction will of course be a product of the specific activity of the component times the concentration. It is perfectly possible for a fraction to have a high specific activity, but a total concentration so low as to make detection exceedingly difficult. Indeed, such properties must be just those expected of an active intermediate. Furthermore, phosphatidic acids are very unstable compounds, as shown by Olley (133), and their extraction from tissues in minute amounts and separation from other lipides present in much higher concentration may well require special methods.

Enzymatic dephosphorylation of phosphatidic acids.—The finding by Weiss, Smith & Kennedy (134) of an enzyme in chicken liver which rapidly dephosphorylates phosphatidic acids with the production of orthophosphate and D-1,2-diglyceride [Reaction (*c*)] offers an explanation both for the metabolic function of phosphatidic acid and the failure of these compounds to accumulate in tissues. The D-1,2-diglyceride produced by the action of this enzyme may then react with cytidine diphosphate choline to form lecithin (134) or with long chain fatty esters of CoA to form triglyceride [Weiss & Kennedy (135)]. The asymmetrical phosphorylation of glycerol in Reaction (*a*) and its dephosphorylation in Step (*c*) produces a diglyceride of the proper D configuration to react with cytidine diphosphate choline to yield a glycerophosphatide of the L series. It is known that the naturally occurring glycerophosphatides are of the L series [Baer (136)].

Phosphatidic acid phosphatase is strongly inhibited by low concentrations of Mg ion (134), a fact which may account for the accumulation of phosphatidic acid *in vitro* in experiments in which this cation was added to the incubation medium.

Phosphorylation of choline and ethanolamine.—The first step in the sequence leading to the introduction of the organic base into the glycerophosphatide is phosphorylation at the expense of ATP [Reaction (*d*)]. Wittenberg & Kornberg (137) described an enzyme present in liver and in yeast which catalyzes the phosphorylation of choline; the purified enzyme also phosphorylates ethanolamine, but only at relatively high concentrations of this substrate. Dawson (138) and Ansell & Dawson (139) have presented evidence that the phosphorylcholine and phosphorylethanolamine of liver and of brain arise by synthetic pathways, rather than by degradation of phospholipides. The concentration of phosphorylcholine in tissues has also been measured by Dawson (140).

Function of cytidine coenzymes in the biosynthesis of phospholipides.—In 1952, Kornberg & Pricer (141) reported in a preliminary communication that phosphorylcholine labeled with P^{32} and C^{14} could be converted by a rat

liver enzyme system to a phospholipide (which was not further characterized). The ratio of P^{32}/C^{14} in the lipide product closely approximated that of the labeled phosphorylcholine, indicating that the molecule had been incorporated as a unit. These results were confirmed by Rodbell & Hanahan (142) who identified the radioactive phospholipide as lecithin after isolation on alumina.

It has been suggested as a working hypothesis that phosphorylcholine might react with a phosphatidic acid, with the elimination of the phosphorus of the phosphatidic acid as inorganic orthophosphate (143, 144). However, no such reaction could be found by Kennedy & Weiss (145, 146) who found instead that the incorporation of phosphorylcholine into lecithin in a particulate enzyme system from rat liver, was activated by amorphous ATP but not by crystalline ATP. The activity of amorphous ATP was traced to contamination with small amounts of the true cofactor, CTP. This discovery marks the first appearance of a cytidine coenzyme as a cofactor in a reaction of major metabolic importance.

It has now been firmly established that a coenzyme form of choline, CDP-choline (Figure 2), is an essential intermediate in Reactions (*e*) and (*f*). A

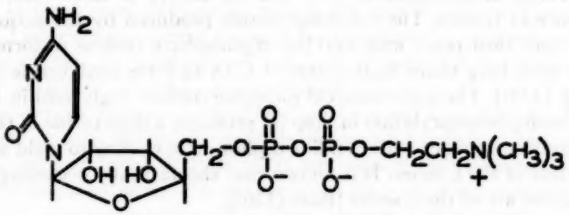


FIG. 2. Cytidine diphosphate choline.

similar coenzyme form of ethanolamine, CDP-ethanolamine, is likewise an intermediate in analogous reactions leading to the formation of phosphatidyl ethanolamine (146). Three separate lines of evidence indicate that the function of the cytidine coenzymes is as shown in Figure 1.

First, synthetic CDP-choline and CDP-ethanolamine, prepared by condensation of phosphorylcholine or phosphorylethanolamine with CMP in the presence of N,N′-dicyclohexylcarbodiimide (147), have the properties required of intermediates in Reaction (*f*). The synthetic compounds are converted to the corresponding phosphatides in high yield and at rapid rates by particulate enzymes from liver (146). The rate of synthesis of lecithin from labeled CDP-choline was much higher than from corresponding amounts of CTP plus phosphorylcholine. When the incorporation of phosphorylcholine-P^{32} was studied in the presence of a bank of unlabeled synthetic CDP-choline, the expected isotope dilution effect was observed, consistent with the conclusion that the nucleotide form is an obligate intermediate.

Furthermore, CDP-choline and CDP-ethanolamine are naturally occurring compounds, present in the liver of the rat and of the hen in appreciable concentrations (146). CDP-choline has also been isolated from yeast as the crystalline sodium salt by Lieberman, Berger & Giminez (148). The compound isolated from yeast has been compared in detail with the synthetic material and found to be identical.

Finally, enzymes catalyzing Reactions (*e*) and (*f*) have been found widely distributed in nature, in such varied sources as liver, kidney, brain, yeast, and carrot root (146).

PC-cytidyl transferase.—The name PC-cytidyl transferase has been suggested for the enzyme which catalyzes Reaction (*e*); the enzyme catalyzing a similar reaction leading to the formation of CDP-ethanolamine is called PE-cytidyl transferase (146). These enzymes are completely specific for CTP; ATP, UTP, GTP, and ITP are inactive. The reaction is readily reversible, and the activity of the enzyme can be followed by pyrophosphorylolysis as well as synthesis of CDP-choline. The enzymatic synthesis of CDP-choline and CDP-ethanolamine follows the fundamental mechanism discovered by Kornberg (149) for the synthesis of DPN:

$$\text{Nucleoside—P—P—P} + \text{R—P} \rightleftharpoons \text{Nucleoside—P—P—R} + \text{P—P} \qquad 21.$$

The synthesis of uridine diphosphate glucose (150) and guanosine diphosphate mannose (151) also takes place by the same general reaction.

CDP-choline and CDP-ethanolamine synthesized by cell-free enzyme preparations have been isolated and found identical with the synthetic products (146).

The effect of CTP as cofactor for the incorporation of phosphorylcholine into lecithin has been noted by McMurray *et al.* (152) in homogenates of rat brain, indicating that the same general pattern of reactions found by Kennedy & Weiss (146) in liver also occurs in this tissue. Williams-Ashman & Banks (153) found that the biosynthesis of lecithin in the seminal vesicle of the rat also involves cytidine coenzymes, and demonstrated Reactions (*e*) and (*f*) in cell-free enzyme preparations from this tissue. In view of the large amounts of glycerophosphorylcholine in the secretion of the seminal vesicle, these workers also looked for a direct reaction between glycerol and CDP-choline, but did not observe such a reaction.

Glyceride transferases.—The enzyme which catalyzes Reaction (*f*) has been named PC-glyceride transferase (146). It requires Mg or Mn ions for activity, but is severely inhibited by the Ca ion. It is completely specific for CDP-choline; synthetic UDP-choline, ADP-choline, and GDP-choline are inactive. The requirement for 1,2-diglyceride to furnish the glyceride moiety of the phospholipide appears also to be quite specific, since triglyceride, phosphatidic acid, or lecithin cannot be substituted. It is essential that the added diglyceride be emulsified with a nonionic surface active agent such as "Tween-20" (Polyoxyethylene sorbitan monolaurate) in order to penetrate to the enzyme surface. When supplemented with CDP-choline,

diglyceride and Tween-20, the enzyme catalyzes an easily measurable net synthesis of phospholipide (134).

The CMP formed in Reaction (*f*) may be rephosphorylated to CTP in Reaction (*g*), catalyzed by enzymes present in liver tissue (154) which utilize ATP as phosphate donor. The cytidine nucleotides may thus function in a catalytic cycle, with the net production of one mole of lecithin or phosphatidyl ethanolamine in each cycle.

Common pathways for the biosynthesis of phospholipides and triglycerides. —There has been considerable speculation that the biosynthesis of triglycerides might take place by reversal of lipase action, particularly in view of the clear-cut demonstration by Borgström (155) that the incorporation of free fatty acids into glycerides may be catalyzed by this enzyme. The experiments of Jedeikin & Weinhouse (156), from which it appears that the incorporation of labeled palmitic acid into neutral fat by liver slices may not require a coupled energy source, also offer support to this idea. Nevertheless, it appears rather unlikely that any considerable net synthesis of triglyceride (as distinguished from isotope incorporation or exchange) can take place by reversal of hydrolytic action.

The first good evidence for the operation of a synthetic pathway of triglyceride synthesis, clearly different from reversal of lipase action, was obtained by Tietz & Shapiro (157) who found that the incorporation of labeled fatty acid into neutral fat in liver homogenates requires ATP and some heat-stable cofactor. When it became apparent that 1,2-diglycerides are essential intermediates in the biosynthesis of phospholipides, Weiss & Kennedy (158) tested the hypothesis that these compounds are also involved in the synthesis of triglycerides and found that a particulate enzyme from chicken liver catalyzes the net synthesis of triglyceride from palmityl CoA and 1,2-diglycerides. The enzymatically synthesized triglyceride was isolated by chromatography on silicic acid, a method based on that of Borgström (159). It is necessary to emulsify the 1,2-diglyceride in a surface active agent to penetrate to the enzyme surface. Palmityl CoA cannot be replaced by equal amounts of palmitate plus CoA in this system.

Since 1,2-diglycerides are now recognized as intermediates in synthesis of both neutral fat and phospholipides, the importance of these compounds in lipide metabolism is obvious. It is of interest that 1,2-diglycerides are also the primary product of the action of pancreatic lipase on triglycerides (160, 161). In Figure 1, free glycerol, L-α-glycerophosphate, and phosphatidic acid are shown as precursors of 1,2-diglycerides, but the existence of alternative pathways *in vivo* must be considered. In particular, it seems unlikely that Reaction (*a*) of Figure 1 is involved in the resynthesis of triglycerides during intestinal absorption, since free glycerol is not incorporated into triglycerides to a significant extent during this process (162, 163). It is possible that L-α-glycerophosphate formed in glycolysis is the precursor of phosphatidic acid and 1,2-diglyceride in intestinal mucosa. Another alternative pathway

might involve a reaction between a 2-monoglyceride and palmityl CoA, yielding a 1,2-diglyceride which may then be converted to triglyceride via Reaction (*h*).

The close interconnection between phospholipides and triglycerides seen in Figure 1 may eventually afford some basis for an understanding of the lipotropic action of choline and inositol in terms of specific enzymatic reactions.

Possible functions of other cytidine nucleotides.—Since CDP-choline and CDP-ethanolamine are precursors of the corresponding glycerophosphatides, it might be expected by analogy that cytidine diphosphate serine and cytidine diphosphate inositol might similarly be intermediates in the biosynthesis of phosphatidyl serine and inositol monophosphatide, but no evidence of the occurrence of these nucleotides in nature has yet been presented. Two nucleotides from *Lactobacillus arabinosus*, originally described by Baddiley & Mathias (164) as cytidine-phosphate-X and cytidine-phosphate-Y, have been more recently identified as the nucleoside diphosphates, cytidine diphosphate glycerol (165) and cytidine diphosphate ribitol (166). The finding of the glycerol compound in particular suggests a function in the realm of lipide metabolism.

The brilliant work of Leloir and his collaborators (cf. 167) led to the discovery of the uridine coenzymes and opened an entirely new field in the metabolism of carbohydrates. It is possible that a curious division of labor has taken place in nature, with uridine coenzymes predominating in carbohydrate metabolism and cytidine compounds in lipide metabolism.

The incorporation of free choline into lipide.—A rapid incorporation of free choline into the lipides of isolated particulate enzymes from rat liver was noted by Kennedy (223). Neither phosphorylcholine nor L-α-glycerophosphorylcholine are intermediates in this reaction, for which ATP and CoA are necessary cofactors (224).

The original identification of the product of this reaction as lecithin (223) has more recently been withdrawn (146, 225). Further work has shown that the labeled product is a choline-containing lipide free of phosphorus, perhaps a fatty acid ester of choline.

Catabolism of Phospholipides

Oxidation of phospholipides.—The idea that phospholipides are somehow involved in fatty acid oxidation has long been entertained, although it is now firmly established that fatty acid esters of CoA or possibly of pantetheine are essential intermediates in this process. An enzyme which catalyzes the formation of CoA esters from free long-chain fatty acids and ATP was described by Kornberg & Pricer (124), but the possibility remains that phospholipides may somehow be directly and preferentially converted to CoA esters in the living cell. To date, no mechanism for the direct conversion of fatty acid O-ester linkages to CoA thioester bonds has been reported; pre-

sumably the equilibrium of such a reaction would be quite unfavorable.

Indirect evidence which implicates phospholipides in fatty acid oxidation was obtained by Artom (168) who found that fatty acid oxidation is impaired in enzyme preparations derived from the tissues of choline-deficient animals. In a more direct approach, Marinetti & Stotz (169) and Rodbell & Hanahan (170) observed an increased oxygen uptake upon the addition of phospholipides to cell-free enzyme preparations from liver, but the enzymatic mechanisms involved remain obscure. Witter, Shepardson & Cottone (171) have studied the enzymatic oxidation of phospholipides and related compounds and claim to have distinguished two different enzyme systems. The use of the name "phospholipide dehydrogenase" by these workers to describe such enzymes may be somewhat premature, since it has not yet been established that the intact phospholipide is in fact the substrate attacked.

Enzymatic hydrolysis of phospholipides.—Purification of phospholipase A from bee venom by chromatography on aluminum oxide has been described by Habermann, Neumann & Neumann (172) who have also separated phospholipase A from the toxic principle of the venom of *Crotalus terrificus terrificus* by chromatography on ion exchange resins (173, 174). Crystalline crotoxin, formerly believed to be a single pure protein with phospholipase A activity, has recently been resolved by Fraenkel-Conrat & Singer (175) into two components after treatment with fluorodinitrobenzene. The crystalline material appears to be a firm complex between an acidic and a basic protein; it is not known which component has the enzymatic activity.

Phospholipase B has been shown by Dawson (176) to be present in rat liver. Unlike phospholipases A (177) and C (178) this enzyme is inhibited by ether. It is also labile to heating at 55°.

An enzymatic method for the preparation of L-α-glycerophosphorylcholine, using the phospholipase B of *Penicillium notatum*, has been worked out by Uziel & Hanahan (179). Further evidence that L-α-glycerophosphorylcholine and L-α-glycerophosphorylethanolamine are products of hydrolysis rather than intermediates in the biosynthesis of phospholipides has been obtained by Dawson (180). Values for the concentration of these esters in tissues are given by Schmidt *et al.* (181).

A diesterase present in rat liver cleaves L-α-glycerophosphorylcholine to yield L-α-glycerophosphate plus choline [Dawson (182)]. Glycerophosphorylethanolamine is also attacked by this or a similar enzyme.

The enzyme which cleaves phospholipides to phosphatidic acid and free base (lecithinase C) has been studied in plant plastid preparations by Kates 178, 183) who has found that the spinach chloroplast enzyme is not only active in the presence of diethyl ether, but shows virtually no activity in the absence of this solvent (178). Kates has also outlined a useful enzymatic procedure for the preparation of phosphatidic acid from pure lecithin (183). Tookey & Balls (184) have prepared lecithinase C (called lecithinase D in the nomenclature of these authors) in soluble and partly purified form from

cottonseed, and have compared the properties of the cottonseed enzyme with the enzyme from cabbage. The soluble cottonseed enzyme is not activated by ether.

The breakdown of diphosphoinositide in suspensions of guinea pig brain has been investigated by Rodnight (185). Both organic P and inorganic P, as well as inositol, are released into the acid soluble fraction. Maibauer (186) and Maibauer & Herken (187) have studied the release of inositol by nerve tissue, using microbiological methods for the determination of inositol.

Metabolic Functions of Phospholipides

The principal theories on the metabolic functions of phospholipides have been admirably reviewed by Beveridge (188).

Phospholipides and enzyme secretion.—The studies of Hokin & Hokin on the effect of cholinergic drugs on the rate of turnover of phospholipides *in vitro* have likewise been reviewed by these authors (132). The administration of acetylcholine to slices of pigeon pancreas incubated with P^{32} causes a dramatic increase in the specific activity of the phospholipides of the slice, but not in total amount. When the phospholipides are analyzed by the valuable method of Dawson (129) it is found that the highest specific activities are in the phosphoinositide and "phosphatidic acid" fractions, with the greatest percentage increase, upon the addition of acetylcholine, in the phosphoinositides (189). Similar results may be obtained with slices of brain cortex (190) and other tissues (132). In contrast to the results with P^{32}, little stimulation of the incorporation of glycerol into phospholipides occurs upon the addition of acetylcholine, consistent with the observation that no net increase in phospholipide takes place. The effect of cholinergic drugs on phospholipide turnover in pancreas slices is related to secretion of enzymes, although a perfect parallelism does not always obtain (132). In support of this hypothesis, pancreozymin, which stimulates secretion of enzymes by the pancreas, has an effect similar to acetylcholine, whereas secretin, which stimulates the secretion of water and bicarbonate, does not have such an effect (191). The recent report by Hokin & Mazurkiewicz (192) that some effect of acetylcholine may be observed in cell-free preparations may perhaps open the way to localization of the site of action of acetylcholine on some specific enzymatic reaction.

Function of lipides in electron transport systems.—Enzymes catalyzing the terminal oxidation of fatty acids and Krebs cycle intermediates, as well as coupled phosphorylations, are known to be localized in the mitochondria, intracellular particles rich in lipide, particularly phospholipide. There have been many reports in the literature which indicate that destruction of the phospholipide component of the mitochondrial lipoprotein complex by treatment with phospholipases inactivates multienzyme oxidase systems, such as succinoxidase (193 to 197) or octanoic oxidase (198). This has led to the suggestion that phospholipides may play some functional part in electron trans-

port mechanisms. Further support of this hypothesis has recently been brought forward by Tookey & Balls (199) who found that succinoxidase from beef heart is inhibited by cottonseed phospholipase C. These workers obtained evidence that it is the destruction of the phospholipide rather than accumulation of toxic end-products which is inhibitory. Polonovski & Gay (200), in an investigation of the effect of cationic detergents on succinoxidase, also emphasize the importance of the lipide components.

In a more direct demonstration of the role of lipides in electron transport systems, Nason *et al.* (201 to 203) extracted the particulate DPN-cytochrome-*c* reductase of rat skeletal muscle with isooctane, and found a 90 per cent decrease in activity, which could be restored by the addition of *d*-α-tocopherol or by adding back the naturally occurring lipide cofactor found in the isooctane extract. The naturally occurring lipide is not free tocopherol and is effective at much lower concentrations. Crystalline bovine serum albumin in a final concentration of 1 per cent can completely restore the activity of the isooctane extracted enzyme; this effect is due to a lipide material bound to the serum albumin and extractable with isooctane. The authors suggest that the effect of the lipide cofactor is to maintain the spatial integration of the enzymes in a fashion similar to that described for the succinoxidase system by Keilin & Hartree (204).

The content of tocopherol in succinoxidase preparations from heart muscle has been measured by Bouman & Slater (205), who find about 1.1 μM per gm. of protein.

Phospholipides and nerve function.—Tobias (206) has found that treatment of lobster axon or frog muscle with phospholipase A or C leads to depolarization and inexcitability. Although lysolecithin, the product of the action of lecithinase A, appeared in itself to be toxic, no such effects of phosphorylcholine and diglyceride were noted, suggesting that in the experiments with lecithinase C it is the destruction of phospholipide itself which brings about the effects noted.

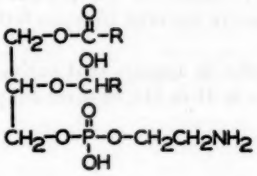
Geiger & Yamasaki (207) report that maintenance of function in perfused cat brain requires the presence of uridine and cytidine in the perfusion medium, possibly for the continued resynthesis of galactosides and phospholipides respectively.

Permeability.—Human red cells treated with phospholipase A (snake venom) maintain glycolysis and are not extensively hemolyzed, but nevertheless lose their properties of selective cation transport, according to Greig & Gibbons (208).

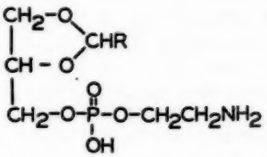
Structure of Native Plasmalogen

Recent work in several laboratories has shown that the compound with the structure shown in Formula I, isolated by Feulgen & Bersin (209) and Thannhauser, Boncoddo & Schmidt (210), is an artefact, and that native ethanolamine plasmalogen possesses a fatty acid residue in ester linkage,

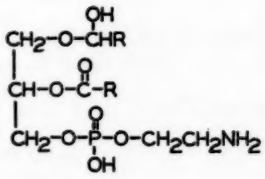
with a structure such as shown in Formula II or III, or some closely related structure.



II



I



III

By avoiding treatment with alkali, Klenk & Böhm (211) in 1951 obtained preparations of ethanolamine plasmalogen with a P content of 3.1 to 3.6 per cent, much lower than that demanded by Formula I. In 1953, Schmidt, Ottenstein & Bessman (212) reported that crude native plasmalogen did not yield acid soluble P upon treatment with fuchsin-sulfurous acid, as did "acetal phosphatide" which had been treated with alkali, and concluded that the alkali-treated materials are artefacts produced by hydrolysis of the native substance. Klenk & Debuch (213) hydrogenated native plasmalogen (not treated with alkali during isolation) and obtained a crystalline product which no longer gave the plasmal reaction, but upon treatment with alkali yielded a mixture of batyl and chimyl α-phosphoric acids. It was not determined whether the ether linkage was in the α' or β position. In more recent extension of this work, Debuch (214) prepared "phosphatidyl ethanolamine" from human brain by the method of Folch (215) and showed that 65 to 70 per cent of the total P of this preparation is present as plasmalogen. An analytically pure "lysocephalin" was obtained from this material by countercurrent distribution after mild treatment with acetic acid at 37°, offering direct evidence for a fatty acid ester group in the native plasmalogen. The fatty acid components of this "lysocephalin" were exclusively unsaturated fatty acids.

The choline-containing plasmalogen of the lecithin fraction of beef heart, detected in 1953 by Hack (216) and by Klenk & Gehrmann (217), similarly yields a lysolecithin containing unsaturated fatty acids [Klenk & Debuch (218)]. Rapport & Alonzo (219) analyzed lecithin prepared from beef heart by the method of Pangborn (220), estimating fatty acid esters as

hydroxamic acids (221) and long-chain aldehydes by the method of Wittenberg, Korey & Swenson (222). The results showed that about two thirds of the total P of these lecithin preparations was present as plasmalogen, and offer further proof that the native material contains fatty acids in ester linkage.

On the basis of these results, it appears that native plasmalogen should be assigned a structure shown in II or III, or some anhydride form, possibly an α,β-unsaturated ether.

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CARBOHYDRATE METABOLISM[1,2]

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Introduction

Increasing pressure is being brought to bear upon the classical framework serving for the description of carbohydrate metabolism, by the constant discovery of new enzymes and alternative metabolic pathways. For this reason, while still referring in the text to such well-recognized and defined routes as glycolysis or the tricarboxylic acid cycle, the reviewers have preferred to order the subject matter of this survey, as much as was feasible, according to the chemical nature of the immediate substrates involved. Such parts of the early degradation pathways as are shared by most sugars have been described under the heading "Metabolism of Glucose."

Faced with the problem of choice, unavoidably imposed by the limitations of space, the reviewers have covered somewhat more sketchily the results obtained on lower animals, plants, and microorganisms, and have given greater attention to the field of their personal experience, mammalian metabolism. Only brief reference has been made to data of purely enzymological interest, which are dealt with more fully elsewhere.

Transport of Sugars

Theoretical considerations.—Recent years have seen a renewal of interest in the mechanisms involved in the transfer of glucose and related sugars across biological barriers. Kinetic derivations have been worked out by Rosenberg & Wilbrandt (1) for a variety of model carrier systems, and by Best (2) and Blum (3) for a process of diffusion followed by enzymatic utilization.

The carrier hypothesis, which is now being considered by an increasing number of workers, usually rests either on the finding that the transfer phenomenon does not obey simple diffusion kinetics, or on metabolic studies, indicating that the utilization of a given substrate shows different properties in intact cells and in cell-free extracts. There is an intrinsic weakness in such

[1] The survey of the literature pertaining to this review was completed in December, 1956.

[2] The following abbreviations are used in this chapter: ADP for adenosinediphosphate; ATP for adenosinetriphosphate, CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPN^+ for the oxidized form of DPN; DPNH for the reduced form of DPN; DPT for diphosphothiamine; Gal-1-P for galactose-1-phosphate; G-1-P for glucose-1-phosphate; GDP for guanosine diphosphate; IDP for inosine diphosphate; P for inorganic phosphate; PP for pyrophosphate; RNA for ribonucleic acid; TPN for triphosphopyridine nucleotide; TPN^+ for the oxidized form of TPN; TPNH for the reduced form of TPN; UDP for uridine diphosphate; UDPG for uridine diphosphate glucose; UDPGal for uridine diphosphate galactose; UMP for uridine monophosphate; and UTP for uridine triphosphate.

arguments, in that they are based on deviations in behaviour from obviously oversimplified models of the cell membrane and contents. They have, however, been considerably strengthened in some cases by the quantitative verification of some of the predictions of the carrier theory. Another point worth emphasizing is that a simple carrier system cannot catalyse the transport of sugar against a concentration gradient, except in a very special case where the carrier is itself involved in the transport of a second component down a concentration gradient and in the opposite direction. This phenomenon has actually been found to occur in one particular case (4).

Single-cell systems.—Park and his co-workers (4) have studied inward and outward transport of glucose across the membrane of rabbit erythrocytes and obtained data which support the carrier hypothesis. The latter is also upheld for human erythrocytes by Wilbrandt *et al.* (5), but is rejected by Mawe (6).

The selective utilization of fructose by certain types of yeasts, possessing a "normal" hexokinase, has been ascribed by Sols (7) to the presence in the cell membrane of a transferring agent with a greater affinity for fructose than for glucose. A specific effect on glucose transport (or on ATP formation) has been postulated by Lampen *et al.* (8) to explain the action of the antifungal Nystatin, which inhibits sugar utilization in intact yeast cells, but not in cell-free extracts. Similarly, the glucose uptake of ascites tumour cells has been found to be inhibited by phloridzin, which is inactive on cell-free extracts, and not by N-acetyl-glucosamine, a potent hexokinase-inhibitor which appears not to penetrate inside the cell (9). Of the other sugar analogues tested on ascites cells by Yushok & Batt (10), most had inhibitory properties which roughly paralleled their affinity for brain hexokinase.

Intestinal absorption.—The case against the hexokinase theory has been strengthened by Sols (11, 12), who has found significant differences in specificity between intestinal absorption and phosphorylating ability. Even more conclusive evidence has been provided by Crane & Krane (13), who have demonstrated the active absorption of 6-deoxyglucose and 1-deoxyglucose, thus eliminating the possible involvement of a phosphorylation in position 6 or 1, as well as a mechanism by mutarotase action, since 1-deoxyglucose cannot undergo mutarotation. The active absorption of 3-methylglucose, which is not utilized in the animal body (14) and is a substrate neither for hexokinase (11) nor for mutarotase (15), argues in the same direction. The evidence discussed above throws some doubt on an explanation put forward by Chanel (16), who has tentatively related the inhibition of glucose absorption under anoxemia to an experimentally observed inhibition of the hexokinase and phosphatase activities of intestinal homogenates at lower pH and under increased CO_2 tension.

The formation of lactate during the intestinal absorption of glucose appears to depend on the conditions of the experiment and on the animal species. With isolated preparations, it is relatively more important in anaerobiosis than in the presence of oxygen (17) and is greater for rat or mouse than

for hamster, guinea-pig, or rabbit intestine (18). While of considerable importance in the isolated rat intestine incubated *in vitro* (18), it represents only an average of 8 per cent of the absorbed glucose in intestinal loops from the same species perfused *in situ* (19) and may be negligible under entirely physiological conditions, according to evidence derived from an analysis of the hepatic glycogen formed from glucose-1-C^{14} (20).

A number of new inhibitors of glucose absorption have been described by Ponz & Lluch (21). Various aspects of the intestinal absorption of sugars have also been studied on isolated preparations by Quastel and co-workers (22, 23) and by Wilson & Vincent (24). These workers have confirmed and extended the earlier observation that fructose is absorbed partly by passive diffusion and partly by an active conversion to glucose. Lactate is also formed during the absorption of fructose *in vitro* (18). These findings raise the problem of the fate of dietary fructose in the intact animal. In a recent study by Kiyasu & Chaikoff (25), performed on isolated jejunal loops left *in situ*, glucose represented the main product of the intestinal absorption of fructose in the hamster; in the rat, between 30 and 70 per cent of the absorbed fructose appeared as such in the portal blood, glucose production was slight, but considerable amounts of lactate and other unidentified products were formed. In unanesthetized rats, however, fructose is not removed from the gastrointestinal tract much more rapidly than 1-methyl-fructose, which is presumably absorbed exclusively by passive diffusion (26).

From a study of the absorption of glucuronic acid and glucuronolactone in the rat, Packham (27) has concluded that these compounds are mostly absorbed in the form of bacterial degradation products.

Tubular reabsorption.—The problem of renal reabsorption of glucose has been examined from the theoretical point of view by Burgen (28), and in relation with the action of phloridzin by Lotspeich and co-workers (29, 30).

Placental transfer.—The transmission of sugars across the placental barrier has been studied in humans and monkeys by Chinard *et al.* (31) and in sheep by Alexander *et al.* (32, 33). The latter authors have also investigated further the formation of foetal fructose from maternal glucose. Goodwin (34) has recently reviewed the species specificity of this process.

Blood-to-tissue transfer and the action of insulin.—The intracellular penetration of sugars under the influence of insulin has been the object of further studies by Park and his co-workers (4, 35, 36, 37). The phenomenon could be evidenced on the diaphragm, heart, and skeletal muscles, but not on the brain, of intact and of eviscerated-nephrectomized normal rats, as well as on the perfused rat heart. A distinctly smaller response to insulin was observed on the heart and diaphragm of alloxan-diabetic rats. In contrast with earlier findings of the Levine school, insulin was found to affect the penetration of all true monosaccharides tested, including D-glucose, D-galactose, D-mannose, D-fructose, D-xylose, L-arabinose, D-ribose, and D-fucose, but not of hexitols, such as mannitol or sorbitol. The effect on pentoses has been confirmed by Helmreich & Cori (38), who have added D-arabinose and

D-lyxose to the above list, and again drawn attention to the fact that stimulation of the muscles produces the same effect as insulin. The latter observation has also been made by Huycke & Kruhøffer (39).

The nature of the transport mechanism involved and the significance of the insulin effect are still debated. Park *et al.* fully endorse the view originally propounded by Levine that the intracellular penetration of metabolizable sugars is the rate-limiting step in their utilization, catalysed by a specific system, the stimulation of which forms the primary effect of insulin. Helmreich & Cori, on the other hand, put forward the alternative hypothesis that increased metabolic activity, as induced either by insulin or muscular work, may alter the properties of the cell membrane in such a manner as to render it more permeable to sugars, some of which, at least, could penetrate by simple diffusion. This view would make the permeability effect synergistic with other metabolic effects, rather than causative of them.

It is not possible, at present, to distinguish between these two hypotheses. Arguing against the carrier theory are the broad specificity of the transfer mechanism, as well as the results of recent competition studies performed on the isolated rat diaphragm by Nakada & Wick (40 to 43) and by Huycke & Kruhøffer (39). These indicate that the rate-limiting step of glucose utilization has a specificity pattern resembling qualitatively that of known hexokinases rather than that of the transfer process. On the other hand, the competitions between glucose and galactose observed by Fisher & Lindsay (44) on the perfused rat heart support the carrier hypothesis. Puzzling with respect to both theories are the observations of Shaw & Stadie (45) showing that insulin influences the formation of C^{14}-glycogen, but not that of labeled lactate, in the isolated rat diaphragm incubated in the presence of C^{14}-glucose.

As an alternative to the phosphorylation and permeability theories, Keston has put forward a modern version of the γ-glucose hypothesis, involving the action of mutarotase. In further support of this theory, Keston (46) advances the observation that the substrates of mutarotase, D-galactose, D-xylose and L-arabinose are all cataractogenic, as also shown by Patterson (47). Competition with glucose for mutarotase is postulated to explain the effect of these sugars. As mentioned above, strong evidence has been adduced against the participation of mutarotase in intestinal absorption. The recent report by Nath & Bhattathiry (48) who claim to have identified 1,2-dienol-glucose in normal, but not in diabetic blood, may be relevant to these problems.

Of interest are the observations by Park *et al.* (4) that fructose and ribose do not pass the blood-brain barrier, whereas glucose and several other sugars not only do so but even appear to penetrate to some extent inside the brain cells. Inward and outward transport of glucose has been studied on liver slices; no effect of added insulin on either process could be detected (49). The concentration of glucose in a number of rat tissues has been measured with a specific method by Gey (50).

Metabolism of Glucose

Alternative pathways of glucose catabolism in animal tissues.—A number of additional papers on the occurrence and relative importance of non-Emden-Meyerhof pathways in animal tissues have appeared. These include investigations on intact animals by Bloom, Eisenberg & Stetten (51), and Arnstein & Keglevic (52); on isolated liver slices by Wenner & Weinhouse (53), Ashmore *et al.* (54), and Marks & Horecker (55); on mammary gland slices by Glock *et al.* (56) and Duncombe & Glascock (57); on tumours and lymphatic tissues by Kit (58); on blood cells by Coxon & Robinson (59); on neoplastic tissues by Wenner & Weinhouse (60); and on arbacia eggs and embryos by Krahl (61).

The methods, of which there are many variants, generally rest on the fact that the operation of the oxidative pathway preferentially removes the first carbon atom of glucose. Exceptionally, the introduction of C-2 in position 1 and its removal by a second oxidative cycle are also considered. The removal of C-1 has been assessed either "positively" through its appearance as CO_2, or "negatively" through its nonappearance in various molecules known to arise from pyruvate, the basis of comparison being provided by the fate of C-6.

It is a general feature of these experiments that, even after apparently suitable corrections have been introduced, the positive methods always furnish higher estimates of the contribution of the oxidative pathway than the negative ones. This, as pointed out by Ashmore *et al.* (54), is true even when the two types of calculations can be made with data from the same experiment. Such discrepancies strongly support the conclusion arrived at by Wood (62) that some of the assumptions underlying these calculations are invalid or oversimplified and that "before the calculations can be considered to have a true quantitative significance further information is needed on the mechanism of the alternate pathway." This opinion is also expressed in various ways in many of the papers quoted above and the tentative nature of the conclusions reached is stressed by most authors.

The experimental results themselves often vary greatly from one investigator to the other. Thus, the reported ratios of $C^{14}O_2$ formed from glucose-6-C^{14} to that arising from glucose-1-C^{14} in isolated liver slices range between 0.2 and 0.8. Another puzzling discrepancy is provided by the results of Bloom *et al.* (51), which, contrary to previous data, appear to indicate a greater contribution of the oxidative pathway in muscle than in liver tissue.

These differences suggest that the mode of operation of this pathway, or its quantitative importance, is easily modified under the influence of various experimental factors and does not represent a biological constant in the usual meaning of the word. Pertinent to this question are the data recently published by Glock & McLean (63), who have applied a newly developed method to the measurement of the levels of reduced and oxidized coenzymes in animal tissues. In all tissues investigated, TPN occurred almost exclusively in the reduced form, whereas DPN was present predominantly (51 to

91 per cent) as DPN^+. To the extent that these result reflects the true intracellular steady state levels of the coenzymes, they indicate that the reoxidation of TPNH occurs more slowly than that of DPNH, a conclusion in agreement with the low TPNH-specific cytochrome-*c* reductase activity of liver tissue (64, 65). The possibility thus arises that direct hexose monophosphate oxidation may be of secondary importance as a catabolic process generating high energy phosphate bonds, and be largely involved in biosynthetic phenomena, as a source either of suitable carbon intermediates or of TPNH. A corollary of this hypothesis is that the rate of the process should depend on the availability of appropriate electron acceptors. That this may indeed be the case is suggested by recent observations showing that addition to the incubation medium of substances such as glyceraldehyde and glucosone, which can act as substrates for the TPNH-linked aldose reductase (see p. 157), increases considerably the release of $C^{14}O_2$ from glucose-1-C^{14} by isolated rabbit liver slices (66).

Isotopic experiments of the type discussed could be greatly vitiated if separate pools exist for the intermediates which are common to the two pathways. The recent work of Newburgh & Cheldelin (67), showing that the enzymes of the oxidative pathway are localized essentially in the nonsedimentable fraction of liver and kidney homogenates, which is known also to contain the glycolytic enzymes, renders this possibility less likely.

Other aspects of glucose metabolism in animal tissues.—The decrease in glucose tolerance after a period of fructose feeding, previously observed on rats, has been found to occur also in dogs (68), but not in man (69, 70).

Several studies have dealt with the metabolic properties of the isolated rat diaphragm. Randle (71), has studied the effect of anaerobiosis on the glucose uptake of this preparation and has obtained evidence for the formation of reducing substances other than glucose. The actual net production of largely fermentable reducing substances has been observed by Martonosi & Issekutz (72) in the presence of 2,4-dinitrophenol. The latter substance inhibited glucose uptake, increased the breakdown of glycogen and the hydrolysis of creatine phosphate. Similar findings have been made with dinitrophenol by Walaas and co-workers (73, 74), who were able to relate the effects observed to a stimulation of the adenosinetriphosphatase activity of the preparation, and with salicylate by Smith & Jeffrey (75), who put forward the hypothesis that this compound may act as an uncoupling agent.

Extracts from skeletal but not from heart muscle have been found by Cori *et al.* (76) to catalyse the transfer of phosphate from phosphoglyceryl phosphate to creatine under conditions which preclude the participation of ATP and of the Lohmann enzyme. Glucose-1-phosphate transphosphorylase has now been described in muscle extracts by Sidbury, Rosenberg & Najjar (77). The enzyme catalyses the reversible conversion: 2 Glucose-1-phosphate$\rightleftharpoons$Glucose-1,6-diphosphate+Glucose. The equilibrium of the reaction is dependent on the concentration of Mg^{++}, which appears to act by trapping glucosediphosphate. In the absence of Mg^{++} and with excess glu-

cose, the formation of glucose-1-phosphate could be demonstrated. Acting in conjunction with glucose-1-phosphate phosphokinase, this enzyme could thus catalyse a transfer of phosphate from ATP to carbon one of glucose, without the participation of phosphoglucomutase and hexokinase.

Previous studies on the specificity of glucose-6-phosphatase (78, 79) have been extended to a number of new compounds by Maley & Lardy (80). The properties of fructose-1,6-diphosphatase, purified from liver by a new procedure, have been studied by Mokrasch & McGilvery (81). Leder (82) has purified hog-kidney gluconokinase and investigated the specificity of this enzyme.

McIlwain, who has recently published a monograph on the biochemistry of the central nervous system (83), has continued his experiments on the effects of electrical stimulation on the metabolism of isolated brain slices. When applied aerobically, the pulses produced a rapid but momentary increase of glycolysis, followed by a sustained increase in respiration (84). Anaerobic stimulation caused a persistent decrease of glycolysis and suppressed the ability of the tissue to respond aerobically to electrical pulses (85). Rat-brain mitochondria have been shown by Gallagher *et al.* (86) to differ from those of liver and kidney in that they can catalyse the anaerobic conversion of glucose to lactate and the complete oxidation of glucose. This difference may be related to the fact that the hexokinase of brain is essentially particle-bound, whereas it is largely soluble in liver and kidney. The activation of rat-brain hexokinase by erythrocyte lysates has been shown by Long & Thomson (87) to be attributable largely to the removal of hexose monophosphate by phosphofructokinase. That the brain of ruminants is equally dependent on blood glucose as that of other mammals and does not utilize circulating acetate has been demonstrated on intact sheep by McClymont & Setchell (88). Prokhorova (89) has studied the renewal of glucose and glycogen in the brain and liver of mice injected with glucose-C^{14}.

The cytotoxic agent 2,4,6-triethylene-imino-1,3,5-triazine has been found by Roitt (90) to inhibit glycolysis and respiration in a number of animal tissues and tumoral cells, but not in yeast. The effect could be related to a stimulation of the breakdown of DPN, which was prevented by nicotinamide and other inhibitors of diphosphopyridine nucleotidase.

In a provocative article, Warburg (91) has restated in categoric terms his theory that cancer cells originate as the result of an impairment of respiration, followed by a compensatory increase in fermentation rate. In this article, and in the further comments which have followed in reply to a criticism by Weinhouse (92), great stress is laid on the properties of pure ascites cells, which show an exceedingly high rate of anaerobic glycolysis.

An interesting type of glycolytic fermentation, leading to the formation of CO_2, acetaldehyde, biacetyl, and H_2S, has been studied by Kun *et al.* (93) on *Endamoeba histolytica* incubated anaerobically in the presence of cysteine. This amino acid is believed to be converted into a labile sulfur compound, possibly β-thiopyruvate, which provides S as electron acceptor for triose-

phosphate dehydrogenase. No requirement for DPN in this reaction could be demonstrated.

Glucose metabolism in plants, molds and bacteria.—Lack of space does not permit individual reference to the numerous papers dealing with the relative contribution of the glycolytic and pentose phosphate oxidative pathways to the catabolism of glucose in plants and microorganisms. Some of the interpretative difficulties which attend such studies have been stressed in a preceding section.

Tagged glucose and ethanol have been used by Milhaud & Aubert (94, 95) to study the kinetics of yeast fermentation. The earliest radioactive intermediates which could be identified were: from glucose-C^{14}, hexose monophosphate, 3-phosphoglycerate, phosphoenolpyruvate, and alanine; and from ethanol-C^{14}, citrate and glutamate.

The mechanism of propionic acid fermentation has been the object of extensive investigations by Wood & co-workers (96 to 99), both on intact cells and on cell-free extracts. Multiple pathways appear to be operative and no clear-cut formulation of the reactions involved has as yet been possible. Of interest is the preparation of a cell-free extract of *Propionibacterium shermanii*, capable of fermenting glucose for many hours and with a net uptake of CO_2, in the absence of added cofactors (99).

Present knowledge of the pathways of glucose degradation in *Pseudomonas fluorescens* has been reviewed by Wood (100). Additional information concerning the nature and fate of the nonphosphorylated products of glucose oxidation in this organism has been provided by the identification of gluconokinase and 2-keto-gluconokinase (101).

Cort *et al.* (102) have observed the accumulation of lactobionic acid in cultures of *Penicillium chrysogenum* grown on lactose, whereas Bean & Hassid (103) have identified in the red alga, *Iridophycus flaccidum*, a carbohydrate oxidase of wide specificity, converting D-glucose, D-galactose, maltose, lactose, and cellobiose into the corresponding aldonic acids, with the simultaneous formation of H_2O_2. These authors have also observed the oxidation of glucose to glucosone in the same organism (104). Evidence for a pathway of glucose degradation over polyacetylenic compounds has been obtained on basidiomycetes by Bu'Lock and Leadbeater (105).

Pasteur effect.—In experiments on extracts of kidney and mammary gland tissue, Terner (106) has obtained further evidence in support of the theory relating aerobic depression of glycolysis to a depletion of phosphate acceptors. However the finding by Stickland (107), who has made a detailed study of the Pasteur effect in intact yeast cells, that propionitrile enhances aerobic glycolysis without inhibiting oxidative phosphorylation does not easily fit with this hypothesis.

As pointed out by Lynen (108), a simple phosphorylation theory of the Pasteur effect is difficult to reconcile with the fact that the hexokinase reaction occurs more slowly aerobically than anaerobically, even in organisms whose hexokinase is not inhibited by glucose-6-phosphate, such as yeast.

Lynen suggests that there may be a shuttle of adenine nucleotides between the mitochondria, which contain the main phosphorylating electron-transport systems, and the extramitochondrial spaces in which the glycolytic enzymes are concentrated. Thus aerobic phosphorylation would cause the adenine nucleotides to accumulate within the mitochondria, thereby depleting the other parts of the cells, including those where hexokinase is operative. That such may indeed be the case has been elegantly shown by Gatt, Krimsky & Racker (109) on isolated systems from rat liver. A reverse Pasteur effect, i.e., an inhibition of respiration by glycolysis (Crabtree effect) could be observed by the addition of a complete glycolytic system to actively respiring mitochondria, provided the ADP concentration was low. With high concentrations of ADP or in the presence of 2,4-dinitrophenol, this effect did not occur, thus indicating that the glycolytic system acted by depriving the mitochondria from essential ADP. The authors also mention the interesting fact that respiring mitochondria may form phosphoenolpyruvate from pyruvate and cause the glycolytic sequence to be reversed. Obviously, self-regulation can only be understood if due regard is paid both to the dynamic and the morphologic properties of the cell, and this may well complicate the interpretation of results obtained on intact cells.

Metabolism of Other Hexoses and Related Substances

Fructose.—The properties of the hexokinase and phosphatases of guinea-pig seminal vesicles have been found by Kellerman (110) to be compatible with the scheme proposed by Mann & Lutwak-Mann (111) for the conversion of glucose to fructose in this tissue. No evidence that this scheme is actually followed in the intact organ has, however, yet been provided. In sheep seminal vesicles, fructose formation has been found to occur by a nonphosphorylative pathway over sorbitol, according to the following sequence (112).

$$\text{Glucose} \xrightarrow{\text{TPNH}} \text{Sorbitol} \xrightarrow{\text{DPN}^+} \text{Fructose}$$

The reactions, which are oriented in the direction of fructose synthesis by the high relative content of the tissue in TPNH and DPN^+ (63), involve, in addition to the previously described ketose reductase, a TPN-dependent system of fairly wide specificity, acting on glucose and on a variety of other aldose compounds such as glyceraldehyde, aldopentoses, glucosone and glucuronolactone. This system, which has also been evidenced in sheep placenta (66), has been tentatively called aldose reductase, but further purification is needed before the involvement of a single enzyme can be accepted. Liver tissue also reduces a number of aldose compounds, though, however, not glucose in the presence of TPNH (66).

Kuyper (113) has published a detailed study of liver ketohexokinase. The enzyme is absent in new-born rats. In adults, it decreases upon fasting and increases after fructose feeding. Hormonal effects and the kinetics of the reaction were also studied.

Specific fructokinases leading to the formation of fructose-6-phosphate have been identified by Medina & Sols (114) in peas, and by Doudoroff *et al.* (115, 116) in fructose-adapted strains of *Pseudomonas saccharophila.* These cultures also utilized mannose after conversion to fructose and metabolized fructose-6-phosphate further by the 2-keto-3-deoxy-phosphogluconate pathway. The isomerase involved in the utilization of mannose has been partly purified by Palleroni & Doudoroff (117), who have studied its specificity. The enzyme also catalyses the transformation of D-lyxose to D-xylulose, of D-rhamnose to D-rhamnulose and of a heptose, presumably D-glycero-D-mannoheptose to sedoheptulose. It is inactive on the phosphorylated sugars and differs from previously described xylose-isomerases (which form D-xylulose from D-xylose) and from the *Escherichia coli* enzyme studied by Green & Cohen (118), which converts D-arabinose and L-fucose to D-ribulose and L-fuculose respectively.

Galactose.—The following nomenclature has been proposed by Kalckar *et al.* (119) to characterize the enzymes involved in galactose metabolism:

(*a*) PGal-uridyl transferase: Gal-1-P + UDPG ⇆ G-1-P + UDPGal
(*b*) Galactowaldenase: UDPGal ⇆ UDPG
(*c*) PP-uridyl transferase: UDPG + PP ⇆ UTP + G-1-P

All three enzymes were found in rat liver but enzyme *a* was not detected in lactating rat mammary gland [(120), see also lactose synthesis, p. 167].

The mechanism of reaction *b*, which shows an absolute requirement for DPN, has been further investigated (121, 122, 123). No isotope was detected in glucose formed from galactose in the presence of H_2O^{18} or T_2O, and this fact has been taken to indicate that a keto intermediate is formed by the postulated dehydrogenation. Enzyme *c*, also called uridinediphosphate-glucose pyrophosphorylase, has been purified 250-fold by Munch-Petersen (124). It does not act on uridinediphosphate-acetylglucosamine or on guanosinediphosphate-mannose.

Kalckar *et al.* (119) have now reported, in detail, their work on the absence of PGal-uridyl transferase from the blood of galactosemic infants, a finding which is corroborated by the observation of Schwartz *et al.* (125) that galactose-1-phosphate accumulates in the erythrocytes of the same subjects.

The galactowaldenase reaction may represent the weaker step in the toxic syndrome which occurs in chickens fed a diet rich in galactose, according to Hansen *et al.* (126), who have observed a distinct accumulation of UDPGal in the livers of these animals.

The utilization of galactose by plants may occur in a manner similar to that observed in animal tissues, according to a study by Hassid *et al.* (127) on canna leaves and wheat seedlings. Galactose was converted to glucose without rearrangement of the molecule and galactose-1-phosphate was identified among the phosphate esters present. *P. saccharophila*, on the other hand, appears to utilize galactose by a pathway which parallels to some extent that involved in the utilization of glucose: oxidation to the

corresponding 2-keto-3-deoxyaldonic acid, which, after phosphorylation, is cleaved into pyruvic acid and glyceraldehyde-3-phosphate (128).

Glucosamine.—The synthesis of glucosamine-6-phosphate from fructose-6-phosphate and glutamine, first demonstrated in *Neurospora crassa*, appears to occur also in *Streptococcus haemolyticus* (129), rat liver (130), and rabbit cartilage (131), according to investigations based primarily on the requirement for glutamine. The previously suggested role of glucosone as an intermediate in the synthesis of glucosamine has been excluded in an investigation by Dorfman *et al.* (132) on *S. haemolyticus*.

The deamination of glucosamine-6-phosphate, to form fructose-6-phosphate and NH_3, has been studied in extracts of *E. coli* by Comb & Roseman (133), in extracts of the same organism and of brain tissue by Faulkner & Quastel (134), and in kidney and liver preparations by Leloir & Cardini (135). The reaction studied by the latter authors was found to be reversible and to require catalytic amounts of N-acetylglucosamine-6-phosphate. The following sequence is postulated:

(*a*) Acetylglucosamine-6-P $\rightleftharpoons$ X
(*b*) Glucosamine-6-P + X $\rightleftharpoons$ Acetylglucosamine-6-P + Fructose-6-P + NH_3

in which X is tentatively assumed to be acetylfructosylamine-6-phosphate. Reaction *b* would then consist in a transacetylation leading to the formation of the unstable fructosylamine-6-phosphate, which decomposes spontaneously. A third reaction is postulated to explain the observed decomposition of acetylglucosamine-6-phosphate:

(*c*) X $\rightleftharpoons$ Fructose-6-P + NH_3 + Acetate

Working with free glucosamine, Faulkner & Quastel (134) noted a requirement for ATP, indicating the necessity of a preliminary phosphorylation of the aminosugar. They also observed in *E. coli*, but not in brain, a stimulation by N-acetylglucosamine, which they relate to an inhibition of phosphatases. In view of the results of Leloir & Cardini, the formation of N-acetylglucosamine-6-phosphate, possibly by a transacetylation reaction, would bear investigating.

Phosphoacetylglucosamine mutase, the enzyme catalysing the interconversion of the 1- and 6-phosphate esters of acetylglucosamine, has been purified from *N. crassa* and studied in detail by Reissig (136). The enzyme is different from phosphoglucomutase, but resembles the latter in its requirement for Mg^{++}, a complexing agent and a diphosphorylated cofactor. At equilibrium, the mixture contains 86 per cent of the 6-phosphate derivative. The enzyme was also detected in animal tissues.

Glucuronic acid.—In a further study of uridine diphosphoglucose dehydrogenase, Maxwell, Kalckar & Strominger (137) have been unable to detect the formation of an aldehyde intermediate. The enzymatic hydrolysis of glucuronolactone by rat liver slices and homogenates has been investigated by Eisenberg & Field (138). These authors observed only a negligible production of $C^{14}O_2$ from the labeled lactone, in contrast with the results of

Rabinowitz (139), who has reported the decarboxylation of glucuronolactone and of glucuronic acid by rat liver and kidney homogenates, fortified with ATP, UTP, DPN, nicotinamide and Mg^{++} ions. The product of this reaction, which may be of interest with respect to pentose formation, was not identified.

Ascorbic acid.—The schemes proposed by Isherwood *et al.* (140) for the synthesis of ascorbic acid have received support from the discovery of enzymes which reduce galacturonic acid derivatives in peas (141) and γ-glucuronolactone in animal tissues (112). The latter reaction has also been observed in guinea-pig liver (68), suggesting that the final oxidation of gulonolactone to ascorbic acid may represent the missing step in this animal. In both types of reduction, TPNH serves as specific electron donor. Since DPN^+ is presumably involved in the oxidative formation of the uronic acids, the biosynthesis of ascorbic acid may be another process favoured by the high relative content of tissues in TPNH and DPN^+. The inhibition of ascorbic acid synthesis by ammonium sulphate in *Phaseolus radiatus* seeds has been shown by Sastry & Sarma (142) to be relieved if glucose is replaced in the medium by glucuronic acid or γ-glucuronolactone.

In contrast with the above results and with previous findings on rats, ripening strawberries have been found by Loewus *et al.* (143) to form mostly 1-C^{14}-ascorbate fron glucose-1-C^{14} and symetrically labeled 1,6-C-^{14}-ascorbate from galactose-1-C^{14}. Glucuronic acid and glucuronolactone were not significantly utilized for ascorbic acid synthesis. Sorbose has been excluded by Burns *et al.* (144) as a possible intermediate between glucose and ascorbic acid in rats.

Rapid and extensive oxidation of the ascorbic acid molecule has been observed in guinea pigs by Burns *et al.* (145) and in rats by Curtin & King (146). In both animals, CO_2 represented the main oxidation product; smaller amounts of diketogulonic and oxalic acid were recovered in the urine.

Hexitols.—Several polyol dehydrogenases have been individualized by Arcus & Edson (147) in extracts of *Acetobacter suboxydans* and of *Candida utilis*, and by Shaw (148) in an airborne organism tentatively identified as a fluorescent species of *Pseudomonas*. The enzymes vary in specificity and other properties; they all oxidize polyols to the corresponding ketoses and utilize DPN as electron acceptor, with the exception of a particle-bound, cytochrome-linked dehydrogenase present in *A. suboxydans*. The production of polyols by osmophilic yeasts has been studied by Spencer & Sallans (149).

Cultures of *E. coli* grown on various polyols contain a number of enzymes which oxidize the polyols and their phosphate esters to the corresponding ketoses and ketosephosphates in the presence of DPN (150, 151). Best characterized among these is a D-mannitol-1-phosphate dehydrogenase, which has been partially purified by Wolff & Kaplan (150). The reaction, which leads to fructose-6-phosphate, has an equilibrium far in favour of mannitol phosphate and may serve as a very sensitive test for fructose-6-phosphate.

An interesting polyol phosphate dehydrogenase, using TPN as cofactor and forming the corresponding aldose phosphates, has been studied by Faulkner (152) in silkworm blood, where it may function in conjunction with the malic enzyme to reduce hexose-6-phosphates. The situation with respect to these polyol phosphate dehydrogenases recalls that observed in mammalian tissues, in which the reversible reduction of ketoses appears to depend on DPN, and that of aldoses on TPN. However, a ketose reductase using TPN as cofactor is also known, namely, the enzyme which reduces L-xylulose to xylitol (153).

Inositol.—The synthesis of labeled myoinositol from glucose-C^{14} has now been observed in the adult rat by Halliday & Anderson (154), while Agranoff (155) has reported the formation and breakdown of inositol by isolated preparations of guinea-pig tissues. Larner *et al.* (156) have extracted from cultures of *A. aerogenes*, a DPN-linked myoinositol dehydrogenase, which may be used for the spectrophotometric determination of the cyclitol.

Metabolism of Pentoses and Related Substances

Biosynthesis of pentoses.—The formation of pentoses from hexoses can occur: (*a*) by the loss of C-1 through the hexose monophosphate oxidative pathway, (*b*) by the loss of C-6 through decarboxylation of uronic acids or their aldonic reduction products (see section on ascorbic acid), (*c*) by molecular rearrangements, as catalysed, for instance, by transketolase and transaldolase. Recent studies by Bernstein (157, 158) indicate that the synthesis of RNA ribose, which has been shown to occur mainly via pathway *c* in the chick, proceeds via pathway *a* in *E. coli* and by a combination of *a* and *c* in the rat. The involvement of pathway *b* could be excluded in these studies, but its participation in other processes associated with pentose formation is indicated by the selective decarboxylation of glucuronic acid and of glucuronolactone observed by Rabinowitz (139), by the appearance of L-xylulose in the urine of animals receiving these derivatives (159), and by studies of arabinose formation in berries (160, 161) and of xylose synthesis in wheat plants (162).

Reactions of free pentoses.—A pentokinase acting only on D-ribose and D-deoxyribose has been purified from calf liver by Agranoff & Brady (163), while Stumpf & Horecker (164) have extracted a specific D-xylulokinase from *Lactobacillus pentosus*. That the latter enzyme may be of fairly general importance is suggested by the presence of enzymes forming D-xylulose from D-xylose in the above microorganism, as well as in *E. coli* (165); from D-lyxose in *P. saccharophila* (117); and from L-xylulose in guinea-pig liver. The latter system has been studied by Touster *et al.* (152, 159), who have shown that mitochondria of guinea-pig liver are able to utilize L-xylulose, after conversion to D-xylulose by the following sequence:

$$\text{L-Xylulose} \xrightarrow{\text{TPNH}} \text{Xylitol} \xrightarrow{\text{DPN}^+} \text{D-Xylulose}$$

As already noted for the orientation of other reversible reactions, the high

tissue levels of TPNH and of DPN^+ will favour the formation of the D-isomer in this system.

A specific L-ribulokinase has been purified from *A. aerogenes* by Simpson & Wood (166), who have observed the further conversion of L-ribulose-5-phosphate to triose phosphate in extracts of this organism. This enzyme may play a role in the utilization of L-arabinose, which is known to be converted to L-ribulose in several microorganisms. Another pathway for the metabolism of L-arabinose, uncovered by Weimberg & Doudoroff (167) in *P. saccharophila*, involves oxidation to L-arabinolactone, L-arabonic acid and α-ketoglutaric acid. This organism also oxidizes D-arabinose to D-arabonic acid, but the latter is further oxidized to 2-keto-3-deoxyarabonic acid, which is cleaved into pyruvic and glycolic acids without prior phosphorylation, unlike the hexoses which follow a similar but phosphorylative route (128). The conversion of D-arabinose to D-ribulose has been described in adapted strains of *E. coli* (118).

In addition to the above reactions, the participation of free pentuloses in transketolase reactions must also be considered. This possibility is denied by Smyrniotis & Horecker (168) for the spinach enzyme but both Srere *et al.* (169) and Kornberg & Racker (170), while agreeing on the nonreactivity of D-ribulose, have reported the formation of fructose-6-phosphate from D-xylulose and erythrose-4-phosphate by yeast transketolase.

Reactions of pentose phosphates.—The phosphoribomutase of bovine uterus has been further studied by Guarino & Sable (171). Like yeast transketolase (169), the liver and spinach enzymes have now been found to act on ribulose-5-phosphate only after the latter's conversion into xylulose-5-phosphate (172). The enzyme involved (phosphoketopentose epimerase) has been purified 500-fold from *L. pentosus* by Hurwitz (173), who has found the equilibrium mixture of the two ketopentose phosphates to contain 60 per cent xylulose-5-phosphate at 25°. Similar results have been obtained by Dickens & Williamson (174), who have studied phosphopentose epimerase and isomerase in muscle, ascites cells, and erythrocytes.

A new reaction of xylulose-5-phosphate, consisting in the phosphorolytic cleavage of the ester into acetyl phosphate and triose phosphate, has been identified by Heath, Hurwitz & Horecker (175) in extracts of *L. pentosus* grown on D-lyxose or L-arabinose. This discovery may bear some relationship to the results of Vandemark & Wood (176), who have obtained evidence of an unidentified splitting of pentose phosphate in *Microbacterium lacticum*.

The existence of other pathways of pentose metabolism, involving pyrimidine nucleotides, is suggested by the recent identification of uridine diphosphate xylose and arabinose in mung bean seedlings (177) and of cytidine diphosphate ribitol in *L. pentosus* (178). The actual formation of the two uridine diphosphate pentoses has been observed by Ginsburg, Neufeld & Hassid (179) in extracts of mung bean seedlings incubated with UTP and α-D-xylose-1-phosphate.

Heptoses.—The biochemistry of heptoses has been reviewed by Bon-

signore *et al.* (180). A bacterial phosphokinase acting preferentially on sedoheptulose has been described by Ebata *et al.* (181). The low activity of such a kinase has been rendered responsible by Nordal *et al.* (182) for the accumulation of the heptulose in *Sedum spectabile.* Sedoheptulose diphosphate has been synthesized by an enzymic method (168).

Tetroses.—A cell-free system utilizing D-erythrose has been prepared from *Alcaligenes faecalis* by Hiat & Horecker (183). The participation of erythrose-4-phosphate appears to occur, but the mechanism by which this ester is formed has not been identified. Extracts of *Propionibacterium pentosaceum* have been found by Shetter (184) to phosphorylate erythritol to erythritol-4-phosphate, an ester which has recently been prepared synthetically by MacDonald *et al.* (185).

Glycolaldehyde.—In an extensive study of the fate of labeled glycolaldehyde, Friedmann, Levin & Weinhouse (186) have found that this compound is converted to glycogen mainly over glycine and serine and does not participate in a transketolase type of reaction.

CO_2 fixation.—In conformity with the established function of xylulose-5-phosphate in the pentose-phosphate cycle, Bassham *et al.* (187) have identified labeled xylulose, but not, however, erythrose, among the phosphatase-treated products of photosynthesis in scenedesmus cells. Milhaud, Benson & Calvin (188) have eliminated hydroxypyruvate as a photosynthetic intermediate.

The pathway leading from ribulose-5-phosphate and CO_2 to 3-phosphoglyceric acid has been further clarified by investigations from the Horecker group (189, 190, 191) and by Jakoby, Brummond & Ochoa (192), who have firmly established the sequence:

$$\text{Ribulose-5-P} \xrightarrow{\text{ATP}} \text{Ribulose-1,5-di-P} \xrightarrow{CO_2} 2\ \text{3-P-glyceric acid}$$

Experiments performed in D_2O-enriched water have provided some foundation for the hypothesis that ribulose diphosphate may react in the form of an enediol intermediate in the carboxylating reaction (193).

The above mechanism of CO_2 fixation probably operates also in chemosynthetic organisms according to investigations by Trudinger (194) and by Aubert *et al.* (195, 196) on *Thiobacillus denitrificans* and evidence has now been obtained by Barron *et al.* (197) that a number of mammalian tissues are able to take up CO_2 anaerobically in the presence of ribose-5-phosphate. Striking activity was observed on tumoral cells, especially on leukemic leucocytes.

With the additional data summarized above, the CO_2 fixation cycle may be completely written in terms of known reactions, with ATP and a reduced coenzyme as energy suppliers. Of particular interest in this respect are the investigations of Arnon *et al.* (198, 199), who have studied the integration within the chloroplasts of the increasingly complex systems catalysing the Hill reaction, photosynthetic phosphorylation and photochemical CO_2 fixation.

Metabolism of Trioses and Related Substances

D- *and* L-*glyceraldehyde.*—Indirect information concerning the metabolism of these compounds may be obtained from studies of the fate of labeled fructose and sorbose, which, after phosphorylation in the 1-position, are cleaved by aldolase to dihydroxyacetone phosphate and one of the glyceraldehyde isomers. As shown by Burns *et al.* (144), L-sorbose-6-C^{14} gives rise to liver glycogen labeled essentially in carbon atoms 1 and 6, with a slight excess of isotope in C-1. The authors assume that L-glyceraldehyde is phosphorylated to L-glyceraldehyde-3-phosphate, which is then converted to dihydroxyacetone phosphate. The latter reaction does not, however, conform to the known specificity of triose phosphate isomerase. It appears more likely that the free triose is first reduced to glycerol and that the latter is then phosphorylated and oxidized to dihydroxyacetone phosphate. The results of Bublitz & Kennedy (200) on the metabolism of labeled glycerol indicate that the form arising from L-glyceraldehyde-3-C^{14} would furnish glycogen labeled in C-1 and C-6. That a similar pathway does not operate to an important extent in the metabolism of D-glyceraldehyde is shown by the results of Muntz (201), who has isolated glycogen with 76 per cent of the label in C-6 and 23 per cent in C-1 from the livers of rats receiving fructose-6-C^{14}. In this case, therefore, direct phosphorylation of the triose by triokinase appears to be the major route of disposal, since a pathway over glycerol would yield 3,4-C^{14}-glycogen.

DL-glyceraldehyde acts as a fairly good hydrogen acceptor for liver alcohol dehydrogenase and this fact has been invoked by Holzer & Schneider (202) to explain the accelerating effect of fructose on the oxidation of ethanol. According to these authors, the previously described glycerol dehydrogenase of liver (203) is identical with alcohol dehydrogenase.

Glycerol.—Further confirmation of the stereochemical requirements of the systems converting glycerol to glycogen has been obtained with synthetic D- and L-glycerol-1-C^{14} by Karnovsky *et al.* (204). Glycerol-1,3-C^{14} has been found by Koeppe *et al.* (205) to give rise in the rat to labeled serine containing 5 times more isotope in C-3 than in C-2. This finding indicates a considerable degree of direct synthesis by way of glycolysis. The phosphorylation of glycerol in kidney homogenates has been studied by D'Abramo *et al.* (206), while Mann & White (207) have demonstrated the active utilization of the triol by spermatozoa. The latter were also found to utilize sorbitol by converting it to fructose.

Phosphoglyceric acids.—Rapoport (208) has provided additional evidence for the contention that the dephosphorylation of 1,3-diphosphoglyceric acid to 3-phosphoglyceric acid occurs largely in erythrocytes by way of 2,3-diphosphoglyceric acid, which is formed by diphosphoglyceric acid mutase and hydrolysed by a phosphatase. The consequence of this detour is that glycolysis proceeds without the net esterification of phosphate.

An interesting thioesterification of glyceric acid by methyl-mercaptan has been studied in yeast extracts by Black & Wright (209). The following steps, catalysed by fairly specific enzymes, were individualized:

$$\text{Glycerate} \xrightarrow{\text{ATP}} \text{3-P-glycerate} \xrightarrow{\text{ATP, } CH_3SH} \text{3-P-glyceryl-methylthiol ester} \rightarrow \text{Glyceryl-methylthiol ester} + \text{P}$$

Neither the mechanism nor the significance of this reaction have been elucidated, but the authors consider the possibility of a cyclic process, in which the ester may act as donor of thiomethyl groups, regenerating glyceric acid.

Metabolism of Organic Acids

Monocarboxylic acids.—Interconversion of lactate, acetol, propanediol, and propanediol-phosphate has been demonstrated with yeast suspensions (210). Small amounts of H_2O_2 and pyruvate have been detected during the oxidation of L-lactate by a purified oxidase preparation from *Mycobacterium smegmatis* (211). Pyruvate oxidation and acyloin formation have been studied in pigeon breast muscle by Juni & Heym (212) and by Paul *et al.* (213), in yeast preparations by Dirscherl & Pütter (214), in *Proteus vulgaris* by Moyed & O'Kane (215), in *Proteus morganii* by Slodki & Kalnitsky (216), in *Staphylococcus aureus* by Wolin *et al.* (217) and in *Lactobacillus arabinosus* by Hunt (218). General features of the numerous systems investigated are the involvement of diphosphothiamine in both types of reactions, as well as the nonrequirement for CoA in acyloin formation and some varieties of pyruvate oxidation. Yeast carboxylase has been found by Dickens & Williamson (219) to catalyse an acyloin type of condensation from two molecules of hydroxypyruvate, yielding erythrulose. The phosphoroclastic splitting of pyruvate has been further investigated on cell-free extracts of *E. coli* by Asnis *et al.* (220).

A DPN-dependent oxidation to diacetyl and a subsequent acyloin condensation yielding diacetyl methylcarbinol are the prominent steps of a cyclic degradation system of 2,3-butanediol to acetate, identified by Juni & Heym (221) in bacteria adapted to grow on the diol. TPN has been found by King & Cheldelin (222) to be four times more effective than DPN in the oxidation of acetaldehyde by a highly purified preparation of the dehydrogenase extracted from *Acetobacter suboxydans.*

Some aspects of the activation of acetate have been studied by Aisenberg & Potter (223) on kidney, liver, and tumour preparations, while Berg (224) has now published a detailed account of his data supporting the involvement of adenyl acetate in this reaction. Similar mechanisms appear to be involved in the activation of various amino acids (224, 225). Activation of fluoroacetate (226) and various aspects of the metabolic effects of fluoroacids (227 to 231) have been investigated.

Two pathways for the utilization of glyoxylate by *E. coli* have been described. One involves the formation of malate by an aldol type of condensation with acetate (232), the other a conversion of two molecules of glyoxylate to hydroxypyruvate by a pathway similar to that leading from glycine to serine (233). The conversion of glycolate to citrate has been studied in an oxalate-decomposing pseudomonas by Jayasuriya (234), and in tobacco leaves by Vickery & Palmer (235).

Dicarboxylic acids.—Sanadi *et al.* (236) have published a detailed account of their experiments showing that GDP is the specific phosphate acceptor in the substrate-level phosphorylation coupled to α-keto-glutarate oxidation. This reaction has been found by Montgomery *et al.* (237, 238) to be strongly inhibited by parapyruvic acid, an impurity of most commercial preparations of pyruvic acid.

A reversible DPN-linked cleavage of succinate to two molecules of acetyl CoA has been demonstrated by Seaman & Naschke (239) in extracts of *Tetrahymena pyriformis*, as well as in a variety of animal and bacterial systems. Lardy *et al.* (240, 241) have investigated further the carboxylation of propionyl CoA to succinate by hepatic mitochondrial preparations. A partly purified pig-heart system catalysing this reaction has been found by Flavin, Castro-Mendoza & Ochoa (242) to form fluorophosphate from fluoride and ATP in the presence of bicarbonate.

A malic dehydrogenase requiring neither DPN nor TPN has been identified by Cohn (243) in *Micrococcus lysodeikticus*. Additional evidence that a nucleotide other than ADP or IDP, presumably GDP (244), is involved in the formation of phosphopyruvate from oxalacetate has been provided by Bartley (245). In a study of this reaction on lamb liver mitochondria, Bandurski & Lipmann (246) have identified a specific inosine diphosphatase (unfortunately not tested on GDP), which they believe may play a role in shifting the equilibrium in favour of phosphopyruvate synthesis in the postulated sequence of reactions leading to this compound from pyruvate, by way of malate. As suggested elsewhere in this review for other reactions involving the two coenzymes, the high tissue levels of TPNH and DPN^+ probably suffice for this purpose, since they should decrease the free energy of the reaction by at least 1500 cal. with respect to the standard value (see pp. 157, 160, 161–62).

The formation of oxalate by *Aspergillus niger* has been investigated by Cleland & Johnson (247) and by Hayaishi *et al.* (248). The latter authors have isolated an oxalacetic hydrase, forming oxalate and acetate from oxalacetate. Injected oxalate has been shown by Curtin & King (146) and by Brubacher *et al.* (249) not to be metabolized by the rat, but to be concentrated to a fair extent in bone tissue. Transacylation with acetyl CoA, followed by a DPT-dependent decarboxylation of oxalyl CoA to formyl CoA are involved in a bacterial degradation of oxalate studied by Jakoby *et al.* (250). Results which support this scheme have been obtained on tuberculosis bacteria by Kimura *et al.* (251). These authors have also studied the degradation of tartrate, a process which has been investigated on *Pseudomonas putida* by la Rivière (252) and on animal tissue preparations by Kun & Hernandez (253) and Kun (254).

Tricarboxylic acids.—An aconitic hydrase, differing from aconitase and yielding citrate exclusively, has been identified by Neilson (255) in *A. niger*. Purified TPN-linked isocitric dehydrogenase has been obtained from pig heart (256, 257). Centrifugal fractionation studies on rat liver have indicated

that the mitochondria: (*a*) are the main site of citrate formation (258); (*b*) contain all of the DPN-dependent isocitric dehydrogenase and only a small, though significant, proportion of the TPN-linked enzyme (259); and (*c*) include a larger proportion of TPN than of DPN, the former being mostly in the reduced form, the latter largely oxidized, as in the whole tissue (260). In addition, evidence has been provided that the DPN-linked dehydrogenase may be the only enzyme involved in the oxidative dehydrogenation of isocitrate (259). These results suggest that the TPN-dependent enzyme may fill in an alternative function, perhaps in supplying TPNH for some reductive processes, as proposed above for the glucose-6-phosphate and 6-phosphogluconate dehydrogenases (see p. 154).

A number of papers have appeared on the recently discovered enzymes citritase and isocitritase, which catalyse the reversible cleavages of citrate to oxalacetate and acetate, and of isocitrate to succinate and glyoxylate (261 to 265). Equilibrium constants, at 26° and pH 7.6, of 1.56 for the first reaction and of 34.3 for the second, have been measured by Smith, Stamer & Gunsalus (265).

General aspects of the tricarboxylic acid cycle.—A highly active Krebs cycle system has been demonstrated in HeLa cells by Barban & Schulze (266). Montgomery & Webb (267) have carried out a detailed study of the factors regulating the operation of the cycle in rat-heart mitochondria. An interesting *E. coli* mutant lacking the condensing enzyme has been studied by Gilvarg & Davis (268). The mutant showed no evidence of an alternative pathway of glucose oxidation, bypassing the tricarboxylic acid cycle. Further proof of the biosynthetic importance of CO_2 fixation by partial reversal of the cycle has been provided by Wiame *et al.* (269).

Metabolism of Glycosyl Compounds

Sucrose.—Contrary to previous negative reports, a sucrose phosphorylase has been detected in sugar beet leaves and partly purified by Pandya & Ramakrishnan (270). The possible involvement of uridine nucleotides in the reaction has however not been tested. Aglucosyl transfer from UDPG to fructose-6-phosphate, but not to fructose, has been found by Burma & Mortimer (271) to be operative in the synthesis of sucrose by sugar cane leaves. Pea extracts, on the other hand, were able to use both fructose and hexose-6-phosphate, as well as several other ketoses, as acceptors for the glucosyl group of UDPG, according to experiments by Bean & Hassid (272).

Lactose.—Gander *et al.* (273) have produced evidence that lactose synthesis in the mammary gland occurs by the transfer of a galactosyl radical from UDPGal to glucose-1-phosphate, yielding lactose-1-phosphate. This scheme is further supported by the finding that the mammary gland contains large amounts of PP-uridyl transferase (120, 274) and galactowaldenase (120), and appears to be devoid of PGal-uridyl transferase (120), an enzyme which might be expected to compete with the transferase forming lactose phosphate (see p. 158). It is, however, not easily reconciled with the labeling patterns

observed by Wood & Schambye (275) on samples of lactose formed from radioactive acetate injected directly into the arterial supply of cow udders. Several β-galactosyl derivatives of N-acetylglucosamine have been identified by Alessandrini *et al.* (276) in crude homogenates of rat mammary gland, bull testis, and rat liver incubated in the presence of acetylglucosamine and lactose. Lactose formation has also been studied on intact cows (277) and on guinea-pig mammary gland slices (278).

Glycogen.—The antagonistic enzymic mechanisms which determine the level of active phosphorylase in tissues have been further elucidated with purified preparations from dog liver (279, 280, 281) and heart (282) by Sutherland and his co-workers, and from rabbit muscle by Krebs & Fischer (283). All three tissues contain enzymes which inactivate phosphorylase with the concomitant release of phosphate, and reactivating systems which reincorporate phosphate into the phosphorylase molecule in the presence of ATP and of divalent ions. In liver tissue, the two reactions appear to be typical phosphatase and phosphokinase reactions, whereas in muscle they are associated with changes in molecular weight of the phosphorylase molecule. The two forms of the heart enzyme, which resemble those from skeletal muscle in their sensitivity to adenosine-5-phosphate, have not been subjected to molecular weight determinations. It has, however, been found that heart phosphorylase can be inactivated and reactivated by the liver systems, and vice versa. When converted to the *b* form by the action of trypsin, which did not cause the release of inorganic phosphate, cardiac phosphorylase could be reactivated neither by the heart nor by the liver kinase systems.

The increase in active phosphorylase produced by epinephrine or glucagon has now been demonstrated on liver homogenates incubated with ATP and Mg^{++} ions (284). These results, together with recent data on glycogen synthesis and breakdown, as influenced in liver slices by glucagon (49) and various cations (285, 286, 287), strongly reinforce the concept that the glycogenolytic activity of hepatic tissue is directly related to its phosphorylase content. This and other puzzling observations have been extensively discussed in a recent monograph by Niemeyer (288), who puts forward the hypothesis that phosphorylase may function exclusively as a glycogen-degrading enzyme and that glycogen synthesis may be catalysed by a different system, possibly uridine-linked. An alternative possibility which could be considered is that the various components which determine the equilibrium of the phosphorylase reaction, as well as the level of activity of this enzyme, are distributed within the cell in a heterogeneous manner such that additional active phosphorylase molecules appear in areas of increasing inorganic phosphate to glucose-1-phosphate ratio. Of interest with respect to these problems are the recent observations showing that the molecular weight of the glycogen units influences their degradation and synthesis, both *in vivo* (289) and *in vitro* (290).

The abnormal glycogen structure observed in two cases of generalized

glycogen storage disease has been related by Illingworth, Cori & Cori (291) to the absence of amylo-1,6-glucosidase.

Glucuronides.—Dutton (292) has demonstrated the transfer of glucuronyl groups from UDPglucuronic acid to a variety of acceptors, catalysed by a microsomal enzyme from mouse liver. The glucuronides formed belonged both to the ether and ester varieties and were found to be of β-configuration. Since the donor molecule is probably of α-configuration, inversion appears to be associated with the transfer, as may also be the case in the mechanism of lactose synthesis. Glucuronyl-transferring activity has been observed by Fishman & Green (293) with β-glucuronidase. Previous work on the synthesis and degradation of glucuronides has been reviewed by Levvy (294). Eisenberg *et al.* (295) have shown that exogenous glucuronic acid or glucuronolactone cannot be activated for glucuronide synthesis in man.

Mucopolysaccharides.—The formation of oligosaccharide chains containing N-acetylglucosamine and glucuronic acid combined as in hyaluronic acid has been observed by Glaser & Brown (296) in extracts of Rous sarcoma incubated in the presence of uridine diphosphate acetylglucosamine, which could be replaced by acetylglucosamine and UTP, and of UDPG and DPN, which presumably served as a source of uridine diphosphate glucuronic acid. Hyaluronidase has been found by Hoffman *et al.* (297) to catalyse the formation of mixed oligosaccharides from hyaluronic acid and sulphated mucopolysaccharides.

The turnover of skin mucopolysaccharides has been studied on rats and rabbits by Schiller *et al.* (298) with the simultaneous use of C^{14}-acetate, C^{14}-glucose, and S^{35}-sulphate. Excessive formation of mucopolysaccharides, mainly hyaluronic acid, has been observed in scorbutic repair tissues by Robertson & Hinds (299).

Other compounds.—From a comparison of the incorporation of glucose-1- and -6-C^{14} into cotton cellulose, Shafizadeh & Wolfrom (300) have concluded that this polysaccharide cannot arise by a simple polymerization process. Two mannans, one radioactive and the other not, have been extracted by Sowden & Frankel (301) from *Torula utilis* yeast grown on glucose-1-C^{14} as the sole source of carbon.

The subject of glycosyl transfers between various oligo- and polysaccharides, which, for lack of space, could not be covered in the present paper, has been reviewed by Edelman (302).

Hormonal Regulations

Insulin.—The mechanism of action of insulin has been discussed in the section on transport. Recent investigations from the Levine and Hastings groups have led to a considerable reappraisal of current ideas on the hepatic action of insulin. The new point of view, which is reflected in two recent reviews (303, 304), is that insulin exerts no immediate action on hepatic

metabolism, comparable to its effect on muscle, but influences the liver mainly by means of slow and possibly indirect modifications of its enzymic pattern. As argued in detail elsewhere (305), such a theory disregards numerous data obtained on intact animals as well as on isolated preparations. It is also contradicted by recent isotopic experiments showing that insulin, (*a*) causes an immediate inhibition of glucose production in the intact dog (306), (*b*) enhances lipogenesis from acetate in the perfused liver from non-acidotic diabetic rats (307), (*c*) stimulates glycogen synthesis from glucose in isolated rabbit liver slices (49), and (*d*) promotes oxidative phosphorylation in rabbit liver homogenates (308).

The existence of direct hepatic effects of insulin does not, of course, exclude the production of long-term influences on the enzymic pattern, and examples of such influences are now available (309, 310). Pyridinocoenzymes are decreased in the liver of alloxan-diabetic animals, as shown by Ricci & Pontremoli (311) and Glock & McLean (312). The ratio of DPN^+ to DPNH was found by the latter workers to be decreased as a result of the diabetic state, contrary to what has been expected by many authors and reported previously by Helmreich *et al.* (313). Such changes, several of which are also brought about by fasting, appear, however, to be consecutive to, rather than causative of the observed metabolic alterations, as is clearly shown by the time-course study of glucose-6-phosphatase made by Ashmore *et al.* (309). The possible importance of acidosis in modifying the metabolic pattern in the liver of diabetic animals is suggested by the observations of Frohman & Orten (314), who have found that the addition of bicarbonate, but not of fructose, to the diet, suffices to correct the defect in the metabolism of acetate.

Several papers deal with the relative importance of the two pathways of glucose catabolism in liver, as influenced by the diabetic state and insulin treatment. As in similar studies conducted on normal animals, striking discrepancies exist between the data obtained by different groups. Confirming earlier results by Bloom (315), Felts *et al.* (316) observe that the hexose monophosphate oxidative pathway is particularly depressed in the liver of diabetic animals and that this defect is reversed and even overcompensated by insulin treatment. Opposite conclusions have been reached by Glock *et al.* (317) and by Ashmore *et al.* (318). The complications involved in such experiments have been discussed in a previous section (p. 153).

Glucagon.—The action of glucagon and epinephrine on phosphorylase has been examined (see p. 168). Both the hormonal nature and the alpha-cell origin of glucagon are still matters for dispute. Of interest in this respect is the report by Fodden & Read (319), claiming the isolation of a glucagon-like polypeptide from canine pancreatic blood, and its increase under the influence of growth hormone or corticotropin. The accumulation of liver glycogen which occurs in rats chronically treated with glucagon or epinephrine has been shown by Costa *et al.* (320, 321) to be a secondary phenomenon, requiring the presence of an intact insulin-secreting system.

Tyberghein *et al.* (322) have described a potentiation by insulin and a few other hormonal and nonhormonal proteins of the glycogenolytic effect of glucagon on isolated liver slices. This phenomenon has been previously shown to occur also with inactivated insulin and several degradation products of this hormone, and to be due to a protection of the added glucagon against an extremely rapid process of inactivation caused by an enzyme system present in liver tissue (284).

Mice suffering from hereditary hyperglycemic obesity, a syndrome believed to be associated with an excessive production of glucagon, exhibit a greatly accelerated turnover of liver glycogen and an elevated level of hepatic phosphorylase, without change of their liver glucose-6-phosphatase or of muscle and kidney hexokinase activities, according to studies by Shull *et al.* (323, 324).

Epinephrine—The increase in the level of active phosphorylase produced by epinephrine has been further studied by Cori & Illingworth (325) both in resting and in stimulated muscles. The accumulation of glucose-6-phosphate resulting from the glycogenolytic effect has been rendered responsible for the inhibition of glucose uptake, by virtue of its inhibiting effect on hexokinase. These effects have been further studied by Walaas (326), who has found, in contradiction to the above hypothesis, that the inhibition of glucose uptake persists in the presence of cyanide, although hexose phosphates do not accumulate. A direct effect on hexokinase was suggested. However, epinephrine caused only a slight inhibition of this enzyme in extracts, whereas adrenochrome, a much stronger inhibitor, does not slow down glucose uptake by the intact tissue (327).

Other hormones.—Experimental hyperthyroidism increases the hepatic utilization of glucose, as measured on the perfused liver (328) and on liver slices (317). It enhances the activities of glucose-6-phosphatase (317) and of the glucose-6-phosphate and 6-phosphogluconate dehydrogenases of liver tissue (329) and decreases its content of pyridinocoenzymes (312).

An increase in hepatic hexose diphosphatase activity following cortisone treatment has been reported by Mokrasch *et al.* (330). Shull *et al.* (331) have observed a very high level of glucose-6-phosphatase in the liver of mice bearing corticotropin-secreting tumours.

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WATER-SOLUBLE VITAMINS, PART I[1]

(VITAMIN B_{12}, FOLIC ACID, CHOLINE, AND PARA-AMINOBENZOIC ACID)

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This review has been prepared from material available to the author up to about December 1, 1956. In accordance with the limitations of space an exhaustive review was not attempted. An effort has been made to select references which bear on biochemical and nutritional aspects rather than on clinical or descriptive phases of the subjects.

VITAMIN B_{12} AND RELATED SUBSTANCES

The nomenclature of the vitamin B_{12} group continues to grow more confusing as additional analogues come to light. The Section for Biological Chemistry of the International Union of Pure and Applied Chemistry has adopted (1) the general name of "cobalamins" for the B_{12} group, with the modifiers "cyano," "hydroxo," "nitrito," etc., for the appropriate compounds.[2] This leaves no simple way to indicate the nature of the nucleotide base, alterations in which introduce the greatest amount of variety into the important members of the group.

The 1st European Symposium on Vitamin B_{12} and Intrinsic Factor was held in Hamburg in May, 1956. The abstracts of the papers which were delivered at this meeting have been published and furnish a good survey of currently active approaches to the biochemistry of vitamin B_{12} (2).

Structure.—The spectacular developments in structural knowledge of B_{12}, reviewed last year by Pfiffner & Bird (3), leave little of comparable significance to be expected this year. The detailed investigations required to confirm or to modify the postulated structure will doubtless require several years of research in a number of laboratories.

Research into the chemistry of the nucleotide moieties of various members of the B_{12} group is progressing by two main routes. Isolation of new

[1] The following abbreviations are used in this chapter: ADP and ATP for adenosinediphosphate and adenosinetriphosphate respectively; B_{12} and B_{12b} for vitamins B_{12} and B_{12b} respectively; CF for citrovorum factor; CoA for coenzyme A; DNA for desoxyribonucleic acid; DPN for diphosphopyridine nucleotide; FIG for formiminoglycine; IF for intrinsic factor; PABA for *p*-aminobenzoic acid; PGA for pteroylglutamic acid; Pi for inorganic phosphate; RNA for ribonucleic acid; and THFA for tetrahydropteroylglutamic acid.

[2] The author has been advised by the President of the Section for Biological Chemistry that proposals for a revised nomenclature, originating in the Hamburg Symposium, have been submitted to the Section for official action. They will be considered by the Commissions on Nomenclature of the Section for Biological Chemistry and the Section for Organic Chemistry in 1957.

factors from natural sources continues to provide new nucleotides whose structures must be determined by classical chemical methods. On the other hand some workers are studying the linking of substitute nucleotides or bases of known structure to Factor B ("etiocobalamin") in resting cell suspensions. This procedure frequently leads to the production of new members of the B_{12} group, although these do not always contain the starting base in unmodified form.

Shunk *et al.* (4) have identified the Factor III nucleoside base as 5-hydroxybenzimidazole. Some evidence which indicates that the sugar is linked as an α-D-ribofuranoside was also presented.

Biosynthesis.—Dellweg, Becher & Bernhauer (5 to 8) have published an extensive series on the biosynthetic analogues of B_{12} which are produced by resting cells of *Escherichia coli* acting upon various substrates together with Factor B. Crystalline products which contain in the nucleotide portion of the molecule, benzimidazole, 5-methylbenzimidazole, 5,6-diethylbenzimidazole, and 5,6-dichlorobenzimidazole, were obtained. These compounds all showed high activity for the test organisms *Lactobacillus leichmannii*, *E. coli*, and *Ochromonas malhamensis*. Among the bases tested which did not appear to yield new derivatives were 4-methyl-, 4,5-dimethyl; and 4,5,6,7-tetramethylbenzimidazole. Some evidence was obtained that 5-chloro-, 6-methyl, 5-nitro-, 5-amino-, and 5-hydroxymethyl benzimidazole were converted to new derivatives, but these were not obtained in crystalline form. In general it was found that substitution in the 2-position of benzimidazole of a methyl, phenyl, amino, or hydroxy group hindered the utilization of the base, while carboxyl or carboxamide groups in the 2 position were eliminated during the condensation. The free bases were found to be very much better precursors of the cobalamins than were the nucleosides or nucleotides. Fantes & O'Callaghan (9) have prepared analogues of vitamin B_{12} from the metabolite fluid after fermentation by *Streptomyces griseus* in the presence of bases but without added Factor B. The 5,6-dichloro-benzimidazole derivative was readily obtained from this base but not from the corresponding diamine. The 2,3-naphthimidazole and 5-amino benzimidazole analogues were also obtained from the corresponding bases. Of considerable interest is the fact that the dichloro- and naphthimidazole analogues were both found to be active in the treatment of pernicious anemia patients.

The synthesis of vitamin B_{12} and similar substances by *Streptomyces olivaceous* was investigated by Ganguly & Roy (10). Orthophenylenediamine when added to the medium was found to stimulate production of compounds with B_{12} activity. A compound was obtained with a lower R_f than B_{12} on paper chromatographic separation. The possibility that this compound is the benzimidazole derivative was discussed.

Three new factors which could not be identified with available known compounds were isolated from sewage sludge by Rábek and co-workers (11). The paper electrophoretic behavior of one of these compounds indicated that it contains a nucleotide but the other two probably do not. A

microbiological investigation of cod liver for vitamin B_{12}-like activity was made by Truscott & Hoaglund (12). The presence of cyanocobalamin, hydroxocobalamin, and the deoxyribosides of thymine, uracil, hypoxanthine xanthine, and guanine was demonstrated.

Johnson, Bentley & Moxon (13) have studied the biosynthesis of Co^{60}-vitamin-B_{12} and several related factors by sheep rumen micro-organisms both *in vivo* and *in vitro*. Seven substances were found to contain Co^{60} and to promote the growth of *L. leichmannii* in the vitamin-B_{12} assay. About 35 to 60 per cent of the vitamin activity was present as vitamin B_{12} and B_{12b}. The remaining substances were not identified. A reddish-brown crystalline pigment which resembled but was not identical with B_{12} was isolated from grasshopper eggs and was found to stimulate red blood cell production in mice rendered anemic with phenylhydrazine [Burgess, Clark & Rolfe (14)].

Properties.—Little work on the chemistry of vitamin B_{12} itself has appeared since the elucidation of its structure. The magnetic susceptibility of a weakly greenish reduction product of the vitamin was measured by Grün & Haas (15) by employing a new micromethod. The largest value obtained, 6000×10^{-6} c.g.s. units per mole, corresponds to about 4 Bohr magnetons and is compatible with the presence of divalent cobalt in the reduced product. The method although not fully described as yet, should be of special interest to biochemists since it permits susceptibility determinations on paramagnetic substances in the range of 0.1 to 0.5 mg. of material.

Bauriedel, Picken & Underkofler (16) utilizing a short time dialysis procedure, studied the binding of vitamin B_{12} and B_{12b} with various substances, including proteins, nucleic acids, and materials of carbohydrate origin. In their experience, vitamin B_{12b} was much more reactive than vitamin B_{12}. Several protein preparations of diverse origin bound B_{12b} in large amounts. On the other hand, a gastric mucosal extract bound approximately equal amounts of the two analogues. In the latter case the cyano group of B_{12} was not released but remained bound in a photolabile position. These authors caution against misinterpretations due to photoconversion of B_{12} to B_{12b}. They found, for instance, that in contrast to reports reviewed earlier (17) B_{12b} and not B_{12} is bound by heparin and by nucleic acid. Filter paper and cellophane were also found to render B_{12b} undialyzable. They concluded, therefore, that little structural specificity was required to bind the B_{12b} analogue.

The copper-promoted decomposition of vitamin B_{12} in solutions of ascorbic acid was investigated by Rosenberg (18). Cupric ion was found to promote strongly the decomposition of the vitamin in ascorbic acid solution, and kinetics of the process indicate that several reactions take place. The cupric ion is inactivated during the course of the destruction of B_{12}.

Molnár & Kelemen (19) reported on mixtures of solvents which are superior to the usual eluting mixtures for removal of vitamin B_{12} and its complexes from charcoal adsorbates.

Assay.—The availability of several assays, both microbiological and

animal, and their general suitability for most purposes is reflected in a rapidly diminishing literature on this subject. Although it cannot be said that animal assays for vitamin B_{12} are equal in ease and reliability to those for several other members of the vitamin B group, nevertheless there does not seem to be much likelihood for marked improvement at the present time.

The influence of the manner of substitution of test materials into basal diets of either animal or vegetable origin has been carefully studied by Dryden, Riedel & Hartman (20). Certain milk products were found to depress growth when present in either type of ration. The depression was accentuated by including iodinated casein in the ration. Part of the effect was attributed to the presence of lactose in the assayed material, although the protein also appeared to be implicated. The same workers [Hartman *et al.* (21)] have applied their modified assay methods to the determination of the vitamin B_{12} content of milk. The values which were obtained varied from 5.5 to 9.4 μg./l. This range is similar to the highest values obtained by the use of microbiological assays. Comparative data were also presented on the B_{12} content of various milk products.

A procedure for the estimation of vitamin B_{12}-like substances in urine has been given by Baker *et al.* (22). They have compared assays with the three organisms: *O. malhamensis*, *Euglena gracilis*, and *L. leichmannii*. Presumably, *O. malhamensis* values give the true vitamin B_{12}-active compounds, while *Euglena* responds to pseudo-B_{12} and *L. leichmannii* both to pseudo-B_{12} and to deoxyribosides. It is possible to estimate these substances by a differential assay method, assuming that other (unknown) substances which might give responses are not present, since the relative activity of pseudo-B_{12} differs in the latter two organisms.

The responses of *L. leichmannii*, *L. lactis*, *L. acidophilus*, *E. coli*, and *O. malhamensis* to eight members of the pseudo vitamin B_{12} group, as well as to vitamin B_{12} and to Factor B, have been given by Peterson, Hall & Bird (23). *O. malhamensis* responded appreciably only to B_{12}. Different members of the group varied considerably in their ability to promote the growth of the other organisms.

Fantes, O'Callaghan & Goodinson (24) have found varying potencies for desdimethylcyanocobalamin depending on the concentration used in assay. In view of this observation, they have cautioned against stating the relative potencies of two active compounds without specifying precisely the methods used. Conclusions, concerning the identity or nonidentity of two growth factors, based on relative microbiological potencies determined by different techniques may also be faulty.

Burkholder & Burkholder (25) have applied the microbiological assay which uses *E. coli* mutant 113–3 to marine solids. These authors found that boiling estuarine mud with sodium sulfite solution greatly enchanced the extraction of B_{12} activity. On the average, a surprisingly high content, equivalent to 0.328 mg. of vitamin B_{12} per kg. of dried mud, was found.

Vitamin B_{12} Function

Bacteria.—What appear to be the most promising recent advances in the study of vitamin B_{12} function have come from two laboratories. Helleiner & Woods (26) by means of ultrasonic treatment of a methionine- or B_{12}-requiring mutant of *E. coli* have succeeded in obtaining cell-free preparations which respond to B_{12}. In phosphate buffer, in the presence of homocysteine, serine, $MgCl_2$, ATP, and a heated extract of *E. coli*, the dialyzed preparations synthesized small amounts of methionine. The addition of 10 mμg./ml. of cobalamin increased the methionine synthesis about fourfold. The omission of any of the components greatly reduced the yield. This achievement should mark the start of a rapid advance in our knowledge of the intimate biochemistry of vitamin B_{12} function.

Downing & Schweigert (27) have prepared uniformly labeled deoxynucleosides from *E. gracilis* grown with $C^{14}O_2$. *L. leichmannii* was grown in the presence of the C^{14}-thymidine with or without added vitamin B_{12}. The deoxyribose moiety of the thymidine was recovered in undiluted form from the DNA nucleosides of the organism grown without B_{12}. On the other hand, when B_{12} was present in the growth medium the sugars from the DNA contained very little radioactivity derived from the thymidine (28). These results appear to demonstrate that B_{12} is involved more or less directly in the synthesis of deoxyribose in *L. leichmannii*. In those organisms, which can grow without vitamin B_{12} either added or endogenously produced and in the absence of added deoxyribosides, there must be some alternative mechanism for producing deoxyribose. Higher plants may require such a mechanism, as well as may those B_{12}-less mutants which can grow with methionine as a sole substitute for B_{12}. That the full nutritional requirement of *L. leichmannii* for B_{12} is not met by substitution with deoxyribosides is indicated by the findings of Deibel *et al.* (29). When the organism is grown with inadequate B_{12}, filamentous forms result and if thymidine is used to replace B_{12} there are similar growth anomalies.

Dubnoff & Bartron (30) have continued the studies of Dubnoff on the possible relation of B_{12} to sulfhydryl compounds. These authors found several enzyme activities of *E. coli* mutant 113–3 that could be restored after inactivation by aging if treatment with B_{12} and with glutathione were made. These results were interpreted as supporting a general role for vitamin B_{12} in altering the structure of proteins. In related work the same authors have presented evidence that B_{12} activates protein sulfhydryl groups (31). The suggestion was made that after reactivation of the —SH groups by B_{12} the presence of substrate was necessary to prevent reformation of unspecified internal —SH bonds within the protein. The presence of substrate had been found necessary to obtain maximum increases in activity.

In other studies with *E. coli* mutants, Saxena & Agarwala (32) have found that molybdenum, tungsten, or phenylhydrazine inhibition can be competitively reversed by B_{12}. In the case of phenylhydrazine the competi-

tion is exerted even in the presence of methionine. Further interpretation of these findings will be awaited with interest.

Alimchandani & Sreenivasan have reported experiments on the reversal of sulfonamide action in *E. coli* by vitamin B_{12} (33). It was found that B_{12} was superior to methionine in overcoming inhibition, due to sulfanilamide, of an *E. coli* mutant which grows satisfactorily when either the vitamin or methionine is furnished. In accord with expectations, added xanthine or serine spared the vitamin-B_{12} requirement at high inhibitor concentrations. The conclusion was reached that B_{12} assumes additional functions in a condition of stress caused by sulfanilamide.

Animals.—In animal experiments Sreenivasa Murthy, Desikachar & Swaminathan (34) were unable to find any specific effect of vitamin B_{12} deficiency on several rat liver dehydrogenase activities, although there was a general decrease in apparent enzyme concentrations. O'Dell *et al.* (35) observed a marked decrease in cytochrome oxidase in the livers of rats which were newly born from vitamin B_{12}-deficient dams. Neither choline oxidase nor succinoxidase activities were affected.

While the belief that vitamin B_{12} has nothing to do with transmethylation continues to grow, as indicated in last year's review (3), some workers are still concerned with the possibility. Ericson & Harper (36) have studied the effect of diet on betaine-homocysteine transmethylase activity of rat liver. The enzymatic activity was found to be sensitive to the protein content of the diet, increasing rapidly when the protein was increased from a level of 9 per cent to one of 18 or 20 per cent. When B_{12} was lacking in the diet the transmethylase activity declined slowly but never reached values lower than 80 per cent of the controls. However, a cofactor preparation from the livers of chicks, pigs, or rats was capable of increasing the transmethylase activity of deficient rat liver homogenates. A slight effect of B_{12}, added *in vitro*, on the *de novo* synthesis of methyl groups by the homogenates was also observed [Ericson *et al.* (37)], although B_{12} itself could not replace the cofactor in stimulating transmethylase activity. It was pointed out that, while most of the effects noted were slight in degree, this is not out of line with observations on other effects of B-vitamin deficiency.

Wong & Schweigert (38) have found that an uncomplicated vitamin B_{12} deficiency in rats led to a decrease in liver nucleic acids; the deoxyribonucleic acid was affected to a proportionally greater extent. Lack of vitamin B_{12} in rations which contain iodinated casein interfered with liver regeneration after partial hepatectomy as well as leading to decreased ribo- and deoxyribonucleic acid contents. These workers (39) have also reinvestigated the effect of B_{12} deficiency on coenzyme A levels, utilizing rats as experimental animals. As in the case of chicks [Boxer, Ott & Shonk (40)], the deficiency resulted in elevated Co A levels in liver. Wong & Schweigert (38) reported that porphyrin "whiskers" are one of the signs observed in their B_{12}-deficient rats. The work of Allen (41) on protoporphyrin levels in the red blood cells of vitamin-B_{12} deficient sheep may help to explain this finding.

The B_{12}-deficient sheep, in some cases, had red blood cells with ten times the average normal content of protoporphyrin. The high protoporphyrin content of the cells of vitamin-deficient sheep was accompanined by a high plasma iron. In contrast, copper-deficient sheep had high red blood cell protoporphyrin with a lowered plasma iron content.

The synthesis of uric acid from serine-3-C^{14}, methionine-$C^{14}H_3$, glycine-2-C^{14}, sodium formate-C^{14}, and formaldehyde-C^{14} was investigated in normal and B_{12}-deficient chicks by Mistry, Tseng & RamaRao (42). No effect of the deficiency on the specific activity of urinary uric acid was observed with labeled glycine. Reduced specific activity, compared with controls, was observed with the serine, methionine, and sodium formate tracers, while an elevated specific activity was found in the uric acid from deficient birds after feeding C^{14}-formaldehyde. These results were interpreted to mean that B_{12} plays no role in the utilization of formaldehyde, while it is important in the conversion of formate, serine, and methionine to one-carbon precursors of purines. In the absence of data on pool size and total activity, such an interpretation is subject to question, however. Arnstein & Stanković (43), for instance, found that glycine biosynthesis, presumably largely from serine, was not influenced in rats by B_{12} deficiency. Since the cleavage should give rise to formaldehyde or to an active one-carbon fragment at the oxidative level of formaldehyde, it seems probable that this observation is in conflict with the above interpretation.

If the assumption is made that vitamin B_{12} in animal experiments functions solely to promote methyl group neogenesis many diverse observations of its effects in nutrition can be reconciled. Observations which have been made on the accelerating effect of B_{12} on the production of *p*-dimethylaminoazobenzene liver tumors in rats by Day, Payne & Dinning (44), and confirmed by Miller (E. C.) *et al.* (45), are in harmony with the concept. Since the demethylated dye is noncarcinogenic an increase in the available methyl groups should tend to reduce the net rate of demethylation and therefore maintain a higher concentration of the active carcinogen. It is somewhat of a surprise therefore that Bennett, Ramsey & Donnelly (46) have found that B_{12} retards tumor induction by *p*-dimethylaminoazobenzene in their experiments. The clarification of the causes of these differences between the sets of experiments will be awaited with great interest.

Muscle creatine levels in young rats fed supplementary folic acid, B_{12}, and methylating agents, with and without glycocyamine, were reported by Poutsiaka (47). It was concluded that the two vitamins do not permit sufficient methyl group neogenesis on a 12 per cent casein diet to provide for both growth and creatine synthesis.

Antimetabolites of B_{12}.—The complex nature of the B_{12} molecule has made it seem improbable that synthetic compounds suitable for testing as B_{12} competitive analogues would soon be forthcoming. Attempts to prepare analogues of this nature, therefore, have been directed chiefly towards altering the natural vitamin by chemical means. The mono-, di-, and tri-

carboxylic acids which result from mild acid hydrolysis of B_{12} have been converted into a number of amide derivatives by Smith, Parker & Gant (48). Many of these were obtained in crystalline form. Tests for any competitive inhibition exhibited by the compounds were conducted by Cuthbertson *et al.* (49). Microbiological evaluation indicated that the methylamide of the mixed monobasic acids with an inhibition index of 150 was the most active of the derivatives. The dianilides of the dibasic acids were inactive, while the tribasic acid gave a weakly active triethylamide. In rat feeding tests slight activity was found but because of the shortage of material the experiments did not include tests for reversibility with B_{12}.

Other attempts to find specific inhibitors have revolved about the testing of compounds related to the base or to the nucleoside moiety of the B_{12} molecule. Tamm (50) and Tamm, Folkers & Shunk (51, 52), have tested a number of such compounds for their ability to inhibit the multiplication of influenza-B virus in the chorio-allantoic membrane from embryonated chick eggs. Of these, halogenated ribofuranosylbenzimidazoles were much the most active and selective. Whether the action of these compounds is exerted by virtue of their close similarity to the dimethylbenzimidazole portion of B_{12} remains to be ascertained.

The interrelationship of vitamin B_{12} with other vitamins than folic acid has occupied the attention of several groups. The concentrations of vitamins of the B group in hepatic tissues of adult rats on B_{12}-deficient diets were determined by Edwards & Outland (53). Their results were reported as ratios of vitamin to liver nitrogen. Interpretation of these complex experiments must await the accumulation of additional data. In the experiments of Mayfield & Roehm (54), the utilization of carotene by rats was significantly increased when B_{12} was added to certain yeast-containing diets. With other yeasts, or with mixtures of added vitamins, no such influence was observed. In other experiments Potter *et al.* (55) found large increases in the carotenoid content of the skin of chicks following addition of B_{12} to the diet. Deficiencies of thiamine, riboflavin, or pantothenic acid did not impair absorption of vitamin B_{12}, according to Hsu *et al.* (56), while a B_6 deficiency reduced absorption of a test dose of Co^{60}-vitamin B_{12} in rats.

Requirements for B_{12}.—The requirements of white Leghorn chicks for vitamin B_{12} have been given by Miller, Norris & Heuser (57). It was found that the growth of the chicks on a minimal dietary supplement was dependent on the quantity of B_{12} in the yolk of eggs of the breeders. The minimum requirement was set at about 0.25 μg. per 100 gm. of diet when the breeders had sufficient B_{12} for 63 per cent hatchability of fertile eggs. With 83 per cent hatchability the requirement was about 0.125 μg. per 100 gm. of diet (containing 194 kcal. of productive energy), while at an energy level of 240 kcal. it was about 0.16 μg. of B_{12}.

Jaffé (58) has examined the B_{12} nutrition of rats during growth, reproduction, and lactation. Three μg. of vitamin B_{12} per kg. of diet eliminated most of the deficiency signs. Five μg. was equally as effective as 30 μg. of B_{12}

plus 0.2 per cent of added methionine in overcoming the deficiency. Rats raised for 18 generations on B_{12}-limited diets showed no signs of a selection toward resistance to the deficiency. Richardson & Brock (59) studied the effect of large quantities of B_{12} in the diet of pregnant female rats on the survival of the young. At 1 mg. B_{12} per kg. of diet, the highest level tested, no adverse effect was observed. Ten μg. per kg. appeared to provide as good survival and weight gains as did higher amounts. No evidence could be found for unidentified factors required for lactation.

Absorption and Metabolism in Man

Mollin & Smith (60) have given a short but useful review of the pathogenesis of vitamin B_{12} deficiency, in which methods for determining the absorption of B_{12} were discussed. Quantitative data on the relationship between intrinsic factor and the vitamin were also included.

Evans (61) has summarized observations on a number of pernicious anemia patients in which absorption of Co^{60}-labeled vitamin B_{12} was measured. The results were compared with those obtained from similar studies on patients who had undergone gastric surgery, or had idiopathic steatorrhea or structural abnormalities of the intestine. In most cases, gastrectomy was followed by failure of absorption of B_{12} and the failure could be corrected by treatment with intrinsic factor. When megaloblastic anemia accompanied idiopathic steatorrhea impairment in absorption of B_{12} was also found. These patients showed a variable response to intrinsic factor. In three epileptic patients with megaloblastic anemia, absorption of B_{12} was normal. The impaired absorption of B_{12} in several cases of structural abnormality of the intestine was more successfullly treated with antibiotics than with intrinsic factor.

Krevans, Conley & Sachs (62) have also published results of an extensive series of tests with radioactive vitamin B_{12} and indicated the criteria for recognition and identification of deficiency states.

Data on serum levels of vitamin B_{12} in people on vegetarian diets have been brought up to date by Wokes (63). Nyberg & Östling (64) have examined the levels of serum vitamin B_{12} in patients with fish tapeworm (*Diphyllobothrium latum*) anemia. The average value found was about 50 $\mu\mu$g. per ml. while the controls averaged 561 $\mu\mu$g. per ml. No overlap in values was found. While these results support the long held belief that the fish tapeworm somehow makes unavailable the B_{12} of the diet, they do not indicate whether this is because there is interference in absorption or whether the worm itself either uses excessively or destroys the vitamin. In this connection it is of interest that Sadun and co-workers (65) found the growth of an intestinal parasite, *Ascaridia galli*, in chicks, to respond markedly to injectable liver extract even though the hosts showed only moderately the signs of B_{12} deficiency.

The possible benefits of vitamin-B_{12} treatment of Jamaican children on low protein diets were examined by Mackay *et al.* (66). They could find no

effect of supplementation with the vitamin in either weight or height increments. In the same study a slight positive effect of aureomycin treatment on weight was reported. The effects of B_{12}, folic acid, penicillin, and aureomycin treatment were studied in Guatemalan children by Scrimshaw, Landon & Perez (67). In early tests positive results were obtained with aureomycin but later tests proved negative. It was concluded that supplementation of the diet with the antibiotics or with B_{12} and folic acid, alone or in combination, was of no benefit.

A very extensive investigation of serum levels of vitamin B_{12} in pregnancy has been reported by Boger *et al.* (68). Serum obtained from 502 women at various stages of pregnancy was assayed. An unmistakable tendency for a decrease of the serum level with the length of pregnancy was observed. Even during the first trimester the average value was below that established for normal males and non-pregnant females. Saturation studies in which relatively large doses of vitamin B_{12} were given daily by intramuscular injection were conducted by Unglaub, Miller & Goldsmith (69). Significant increases in free and bound B_{12} of serum were reported. Maximum levels of bound B_{12} were lower in the pernicious anemia patients than in the normal controls. No correlation was found between urinary excretion and the levels of free, bound, or total vitamin B_{12} in the serum.

The unusual advantage possessed by vitamin B_{12} that it can be labeled with a gamma ray-emitting isotope is still being extensively exploited. Use has been made of Co^{60}-B_{12} by Meyer *et al.* (70) to determine the distribution of B_{12} by body surface counting. Intramuscular doses of 1 μg. of the labeled compound were administered. After removal from the site of injection during a period of 3 to 4 hours maximal radioactivity was detected over liver, spleen, and left kidney in all cases. In further studies Meyer, Arkun & Jimenez-Casado (71) found no effect of a prior course of treatment with amethopterin. The administration of 50 μg. of nonradioactive B_{12} daily for one week did not affect the distribution of the labeled compound. Glass *et al.* (72) investigated the effect of liver disease on hepatic uptake of Co^{60}-B_{12}. While a number of cases were found in which there was a reduced uptake, unaffected by intrinsic factor administration, it was concluded that an advanced lesion did not necessarily severely impair liver-B_{12} uptake.

Distribution studies on Co^{60}-vitamin-B_{12} have also been conducted in rats, hamsters, mice, and guinea pigs by Miller *et al.* (73). In the rat, after parenteral injection, the kidneys contained more radioactivity, while in the other animals more was located in the liver than in other organs.

The urinary excretion test of Schilling (74) has been used by Rosenblum *et al.* (75) to compare absorption of cyano-, chloro- sulfato-, and nitrocobalamins by humans. The cyano derivative was absorbed much more readily, from four to six times as much radioactivity appearing in the urine as from the other compounds. Similar findings were obtained with rats, by the use of the fecal excretion method.

Careful evaluations of the several procedures for the determination of

intestinal absorption have been made, as well as studies designed to determine the mode of action of intrinsic factor. A simplified fecal excretion test was applied by Halsted, *et al.* (76) to a large number of controls, 13 pernicious anemia patients and 11 patients who had total gastrectomies. The conclusion was reached that the method was entirely reliable, but that the convenience of the "parenteral flushing" test of Schilling (74) was to be preferred for its greater simplicity. Recoveries of B_{12} in the feces of controls varied from 5 to 57 per cent of a 0.5 μg. oral dose, with an average of 34 per cent. In pernicious anemia and in total gastrectomy patients recoveries varied from 78 to 100 and averaged 94 per cent.

Booth & Mollin (77) have found that serum radioactivity after ingestion of Co^{56}-vitamin-B_{12} provides a sensitive and useful assay for absorption of the vitamin. Since the excursion in response is much greater than for the tests in current use, this approach may prove to be the method of choice if the preparation of the Co^{56}-B_{12} can be simplified. It would have been very desirable to have such a preparation available for use in the pernicious anemia patients that were reported by Estren & Wasserman (78) to respond to oral doses of vitamin B_{12} at daily levels of 5 to 16.8 μg. The lack of precision inherent in the fecal recovery method and the lack of sensitivity in the parenteral flushing test are such as to make their use in studies of intrinsic factor in this type of patient unsatisfactory.

Intrinsic factor.—A considerable interest in application of the excretion and absorption tests with labeled vitamin B_{12} to study of intrinsic factor (IF) is still apparent. Stephanson *et al.* (79) have attempted to fractionate human gastric juice by continuous electrophoresis and to assay the fractions in pernicious anemia patients by scintillation counting of the liver. They found the activity to travel with the mucoproteins of the gastric juice, but sharp separations were not obtained. Andresen & Skouby (80) isolated a B_{12}-protein complex after treatment of a commercial preparation of IF with the vitamin. The complex, which gave responses at levels of 1 to 1.4 mg. daily in pernicious anemia patients, experimentally shown to be devoid of IF, was separable into two components. One of these, which comprised 80 per cent of the complex, contained 18 μg. B_{12} per mg. protein but was inactive when given alone. Additional evidence, however, is coming to light which casts some doubt on the specificity of IF from different sources. Holdsworth & Coates (81) have noted that rat stomach extracts facilitated vitamin-B_{12} absorption by isolated sections of rat intestine, while a pig pylorus extract also bound the vitamin but hindered its absorption. Somewhat similar tests have been conducted by Weis (82) who found the binding of B_{12} by intestine to be related to the concentration of the vitamin to which the tissue was exposed. Added gastric mucosa had a variable effect on the degree of binding. Comparative studies on the binding of B_{12} and pseudo B_{12} by gastric juice and by serum were made by Bunge, Schloesser & Schilling (83). Neither pseudo B_{12} nor 5,6-dimethyl benzimidazole competed successfully with B_{12} for the physiologic mechanisms effecting absorption

of cyanocobalamin. However, serum binding was not selective between pseudo B_{12} and B_{12}. It was concluded that binding is a necessary but not a sufficient condition for intrinsic factor activity.

The time relationship of excretion of orally administered labeled vitamin B_{12} followed by a parenteral flushing dose, was investigated by Best, Landmann & Limarzi (84). Peak excretion came between 6 and 12 hr. after the dose of radioactive B_{12}. Following the establishment of the most effective collection periods a series of pernicious anemia patients was utilized in an attempt to correlate assay by hematological response with the urinary excretion test [Best *et al.* (85)]. Data on 34 tests of a single IF concentrate seemed to suggest that excretion of radioactivity was proportional to the logarithm of IF dosage at low or moderate levels. Ellenbogen *et al.* (86) also compared their improved urinary excretion assay for IF with the hematologic response method. It was found that IF, equivalent to two to three daily minimum effective doses, was required to give a satisfactory excretion of radioactivity, i.e., about 7 per cent of a 2 μg. dose. A comparison of the three methods which utilize radioactivity was made on 92 pernicious anemia patients by Bull, Campbell & Owen (87) and the results briefly discussed.

THE FOLIC ACIDS AND RELATED COMPOUNDS

The Section for Biological Chemistry of the International Union of Pure and Applied Chemistry has suggested rules for the nomenclature of the folic acid group of compounds. The term "folic acid" is retained for the pteroylglutamates. The one now known as folic acid, folacin, or vitamin B_c is to be called pteroylmonoglutamic acid. Pteroyltriglutamic acid and pteroylheptaglutamic acid are the names recommended for the acids which contain respectively three and seven glutamic acid residues (1).

Nutrition.—Spray (88) has discussed the role of folic acid and related compounds in macrocytic anemia, particularly the seeming "reciprocal" relation between folic acid and vitamin B_{12}. He concludes that the hypothesis of Vilter (R.W.) *et al.* (89) is still the most plausible one to explain the results of treatment of pernicious anemia with folic acid. Alternative suggestions were not made. This hypothesis has not had wide acceptance among biochemists for obvious reasons. Lear & Castle (90) suggest that folic acid may have a "neuropathic" effect, either directly or by altering vitamin-B_{12} utilization, since neurologic relapses of pernicious anemia patients are much more common with folic acid therapy. They have observed a relative lowering of serum vitamin-B_{12} levels when treatment with 5 mg. of folic acid daily accompanied B_{12} injections. This was interpreted as supporting evidence for an adverse effect of folic acid. In the absence of data on B_{12} balance it would seem, however, that this equally may be considered as evidence that folic acid improves the storage or utilization of B_{12}. A "toxic" effect of excess folic acid has been observed in microbiological experiments by Totter & Gaines (91). The inhibition in growth of *Streptococcus faecalis*, induced by excess folic acid in the presence of large quantities of glycine,

could be partially reversed with the anti-folic acid compound aminopterin. The conditions of the experiment made it seem likely that the adverse effect of folic acid was caused by an overproduction of serine. It seems plausible that if any toxic effect in humans does result from treatment with excess folic acid its cause should be sought in the known normal functions of the vitamin. Recent work by Totter (92) and by Balis & Dancis (93) indicates that divergent pathways of formate metabolism involving folic acid probably occur, one leading to methyl group production, the other to purine production. On this basis one might expect that folic acid would help the pernicious anemia patient by providing a larger reservoir of active one-carbon fragments, a portion of which may be reduced indirectly or directly by any available remaining vitamin B_{12}. At the same time, the unlimited (by B_{12}) production of purines and perhaps of glycine or of serine may have other consequences. RNA synthesis would not be limited by an unsuitable rate of thymidylic acid production, as would DNA synthesis. It has been reported by Kelley, Totter & Day (94) that the feeding of RNA exacerbates fat infiltration into the liver of rats on diets with excess glycine. Serine is also important in phospholipid metabolism. Furthermore, excess purine nucleotide production may well act to withdraw cytidylic acid by RNA synthesis and consequently reduce available cytidine diphosphate choline, and thus reduce phospholipid production. Any or all of these events might contribute to faulty myelin production or otherwise involve nerve function. There is in fact such a multiplicity of possibilities that the offering of hypotheses to explain the interrelationship of vitamin B_{12} and folic acid in the pernicious anemia patient is surely premature.

The rather clean-cut separation of function between folic acid and vitamin B_{12} in relation to nucleic acid precursors is emphasized by the findings of Boxer, Ott & Shonk (40), and Wong & Schweigert (39) that coenzyme A levels are increased in vitamin-B_{12} deficiency. In folic acid deficiency the opposite effect was found by Popp & Totter (95) in accordance with the expectation that all adenylic acid-containing coenzyme concentrations might be low. These divergent results are precisely what should be expected if vitamin B_{12} functions only in the course of reduction of the one-carbon fragments (and perhaps also ribose to deoxyribose) to methyl groups and not generally to provide an extra supply of active one-carbon fragments.

Animal experiments.—The effects of folic acid and of vitamin-B_{12} deficiencies on the synthesis of serine and choline from glycine 2-C_{12} by the liver of turkey poults have been studied by Vohra, Lantz & Kratzer (96). Their results may be compared with those of Totter (97) who conducted somewhat similar experiments with folic acid-deficient chick livers. Results recorded by the former authors give only specific activities without recording total yields, while only total yield data (for serine in the acid-soluble fraction) were given by the latter. Neither experiment provides sufficient data from which to draw firm conclusions. For instance, Vohra *et al.* con-

cluded from the specific activities of the choline and choline methyl groups that there was a slight increase in total methyl group synthesis in the folic acid-deficient liver, whereas there were no differences from the normal in the case of the B_{12}-deficient bird livers. If the data are recalculated, taking into account the final glycine specific activity as a possible measure of the substrate activity during the course of the reaction, it will be seen that there is a 50 per cent reduction in methyl neogenesis from glycine in the livers of the folic acid-deficient birds; this is more in keeping with expectations. On the other hand, a similar calculation with the B_{12}-deficient poult livers indicates that the deficiency led to a doubling of the new methyl formed from the 2-carbon of glycine; such a result is certainly unexpected. Isotopic experiments of this sort require more data than specific activity or total activity before they may be considered fully satisfactory.

Arnstein & Keglević have made a comparison of alanine and glucose as precursors of serine and glycine in the rat (98). With uniformly labeled glucose-C^{14}, after a feeding period of 13 to 20 days, alanine, aspartic acid, glutamic acid, serine, and glycine had similar activities. The labeling in the serine and glycine was essentially uniform. A deficiency in the diet of PGA, but not of B_{12}, specifically reduced the incorporation into glycine of radioactivity from either labeled alanine or labeled glucose but did not affect serine. When labeled alanine was fed, serine and glycine had lower specific activities than isolated alanine, aspartic acid, or glutamic acid. Thus, the pathway from alanine to serine in rats does not appear to be quantitatively as important as that from some nonnitrogenous precursor derived from glucose. Radioactive glycine was fed to rats and guinea pigs by Arnstein & Stanković (43) and the rate of biosynthesis of the amino acid calculated from the specific activity and the analytical data. The rate of synthesis was estimated to be 2.7 millimoles per 100 gm. in the rat and 4 millimoles per 100 gm. animal in the guinea pig. Neither vitamin B_{12} deficiency nor ascorbic acid deficiency (in the guinea pig) reduced the rate. Folic acid deficiency, however, markedly reduced the synthesis of glycine and its conversion to serine and to other amino acids.

Lahiri & Banerjee have also been interested in the possible relationship between folic acid and ascorbic acid. In their experiments they found that an aminopterin-induced "folic acid deficiency" did not prevent the stimulation of ascorbic acid excretion by rats treated with chloretone (99). Tissue stores of ascorbic acid were, in most cases, less in animals which received either aminopterin or sulfanilylguanidine. Chloretone treatment did increase the citrovorum factor excretion in animals receiving folic acid. Guggenheim *et al.* have found an increased excretion of both CF and folic acid after treatment of rats with both PGA and ascorbic acid (100). These workers have also made the interesting observation that phenobarbital treatment has an effect similar to that of chloretone on ascorbic acid and CF excretion. It may be concluded that the precise relationship between ascorbic acid and folic acid is still obscure.

Formaldehyde, labeled with C^{14} and deuterium, as a one-carbon metabolite has been studied in rats by Lowy, Brown & Rachele (101). Ratios of D to C^{14} in methyl groups of choline, creatine, and thymine derived from mixed CD_2O and $C^{14}H_2O$ indicate that formaldehyde was not utilized per se but was first probably converted to formate or to some similar intermediate.

Nelson & Evans (102) have noted the failure of ovarian hormones to maintain pregnancy in rats deficient in either folic or pantothenic acid. Injections of estradiol-17β were found by Mueller & Herranen (103) to stimulate uptake of formate into protein, adenine, guanine and into the acid-soluble serine of rat uterus. The six hours of pretreatment with hormone did not affect formaldehyde utilization under similar conditions. This work again points to a close relation between estrogenic hormones and folic acid. The effect of relatively short deprivation of PGA during fetal development in rats was studied by Nelson *et al.* (104). Straightforward interpretation of the effects noted is somewhat clouded by the necessary use of either antagonists or dietary sulfonamides or both.

An extensive study of the requirement of folic acid by the guinea pig was conducted by Reid, Martin & Briggs (105). These workers have set the minimum dietary level for growth and red blood cell production at the figure of 3 to 6 mg. per kg. For maintaining the leucocyte count the requirement is 6 mg. per kg., or more. These figures should be examined by others in the field of nutrition, especially those who are accustomed to add very small quantities of folic acid to the diet of rats and suppose that the regimen is adequately fortified with folic acid for any contingency.

Requirements of turkey breeder hens were given by Kratzer, Davis & Abbot (106) as a minimum of 0.7 mg. per kg. of feed for optimum hatchability of eggs. This value is only slightly less than the value usually given for optimum growth of poults. March & Biely (107) concluded from studies on diets of natural foodstuffs that the folic acid requirements of chicks is increased on a high protein-high fat regimen.

The blood and urine concentrations of folic acid and other vitamins in the rhesus monkey have been studied by Dutra de Olivera *et al.* (108). No influence of x-irradiation on the excretion of folic acid by the monkey was found in further studies by Dutra de Olivera *et al.* (109).

The very interesting nutritional studies on aseptically reared *Drosophila melanogaster*, published by Sang (110) contain some data on the folic acid requirement of this species.

Microbiological studies.—The necessity in axenic media for folic acid for growth and survival of a strain of *Paramecium aurelia* (Stock 47.8) was shown by Miller & Van Wagtendonk (111). Silverman & Wright (112) have presented data on the microbiological activity of diglutamyl derivatives of CF and of N^{10}-formyl folic acid. Both types of compounds are fully active for *Lactobacillus casei*, while neither is active for *S. faecalis*. The CF derivative showed partial activity for *Leuconostoc citrovorum* (*Pediococcus cerevisiae*). Pancreatic conjugase did not release full activity but digestion

with liver converted the derivatives into fully active compounds. Toennies, Usdin & Phillips (113) have shown that blood hemolysates contain more folic acid activity than is found in plasma and red cells separately. The increased activity appears to be due to the liberation of "bound" folic acid in red blood cells by an enzyme present in plasma. The active compounds were partially purified (114) but do not appear to be identical with well known members of the folic acid group. All six of the compounds found are characterized by relatively high potency for *L. casei* and low potency for *L. citrovorum* and *S. faecalis*. In this respect at least, there is resemblance to those studied by Silverman & Wright (112).

Enzymatic interconversions.—Albrecht & Broquist (115) and Silverman & Gardiner (116) have presented arguments for the occurrence of N^{10} formyltetrahydrofolic acid in human urine after doses of folic acid. This compound is heat labile and gives rise to N^5-formyl-tetrahydrofolic acid (CF) during autoclaving. More recently, Silverman, Ebaugh & Gardiner have identified the N^5-N^{10} bridge compound (anhydro CF) as the quantitatively most important metabolite; the majority of the remaining activity is present as the N^{10}-formyl derivative (116a). Presumably also, according to Albrecht & Broquist, some CF is present in a preformed state, and therefore probably arises by a physiological process.

Doctor & Awapara (117) have reported on the incorporation of carbon-3 of serine into CF. In further studies, Doctor (118) has characterized the enzyme activity in chick liver extracts which brings about this exchange of the methylol group and the subsequent oxidation to formyltetrahydrofolic acid. Homocysteine and Mg^{++} are required. The effects of purine analogues and of folic acid analogues were also tested.

Dinning, Sime & Day (119) and Futterman (120) have reported brief studies on the cleavage of folic acid to a diazotizable amine after incubation with liver slices or extracts. In extracts (120) inactivated by dialysis, activity was restored by adding DPN, ATP, Mn^{++}, and citrate. Aminopterin inhibited the cleavage. Citrovorum factor was also cleaved (119) and the cleavage was not inhibited by aminopterin. The results are entirely consistent with the possibility that, following reduction to tetrahydrofolic acid either an enzymatic or non-enzymatic split occurs, giving rise to *p*-aminobenzoylglutamic acid and a pteridine moiety. The rate of the reaction is such that it should be seriously considered as playing an important part in affecting the net amount of CF or its labile precursor in experiments designed to measure enzymatic "synthesis" of these compounds in liver preparations.

Glycine-serine interrelationship, formate and formimino group transfers.—Wright & Stadtman (121) and Wright (122) have published more details concerning the production of glycine from serine in extracts of *Clostridium* HF. The outlines of this work were fully covered in last year's review (3). Davis, Passonneau & Totter (123) have given a brief report on the exchange of the 3-carbon of serine to glycine catalyzed by extracts of a luminous cocco-

bacillus. The extracts were resolved for cofactor by treatment with an anion exchange resin. THFA substituted for the natural coenzyme only in amounts stoichiometric with the serine. Only formaldehyde or carbon-3 of serine acted as sources of the transferable carbon. Pyridoxal or its phosphate was required but requirements for other factors were not found. This enzyme preparation may offer advantages for some studies since it appears to be devoid of oxidative or reductive ability insofar as the one-carbon fragments are concerned. Serine aldolase was prepared from sheep liver and partially purified by Alexander & Greenberg (124). This preparation did not lend itself to further purification, but it was possible to determine the apparent Michaelis constant for the reaction and to study the equilibrium, approached from both sides. A value for the constant [Serine]/[HCHO][Glycine] of about 2.76×10^3 was found. The reaction required THFA and pyridoxal phosphate.

Huennekens *et al.* (125) prepared serine aldolase from acetone-dried beef liver. This preparation converted serine to glycine and formaldehyde. The cofactor requirements differ from others in that THFA was not required.

The discovery of a tetrahydrofolic acid-linked formimino group transfer by Sagers *et al.* (126) and by Miller & Waelsch (127) was quickly followed by the publications of Rabinowitz & Pricer (128, 129) who have helped to clarify these enzymatic reactions. Formiminoglycine, formed as an intermediate in the breakdown of xanthine by *Clostridium cylindrosporum* is converted by properly prepared extracts from *Clostridium acidi-urici* to glycine, ammonia, and formate in the presence of THFA (126). According to Rabinowitz & Pricer (129) this reaction occurs in at least two steps:

$$\text{FIG} + \text{THFA} \rightarrow \text{glycine} + \text{NH}_3 + \text{N}^{10}\text{ formyl THFA}$$

$$\text{N}^{10}\text{ formyl THFA} + \text{ADP} + \text{Pi} \rightleftarrows \text{HCOOH} + \text{THFA} + \text{ATP}$$

With substrate amounts of N^{10}-formyl-THFA, formiminoglycine is not required for ATP formation. ATP must be removed to allow the reaction to proceed to the right. This latter observation is in accord with those of Goldthwait, Peabody & Greenberg (130) who have published detailed reports on the biosynthesis of glycinamide ribotide and its formyl derivative. The formylation in this case also required formate, a folic acid derivative, and ATP.

Miller & Waelsch (127) have studied the analogous displacement of glutamic acid from its formimino derivative by tetrahydrofolic acid. Kraml & Bouthillier (131) have presented evidence which indicates that formiminoglutamic acid is a quantitatively important intermediate in histidine breakdown in the rat. Transfer from this compound may provide a mechanism for the direct incorporation of the formimino group into an aminoimidazole residue, resulting in a biosynthesis of guanine derivatives independently of the hypoxanthine route. If this new route should prove to be of importance in the synthesis of purines it may at last justify at the enzymatic level the conclusion reached by Rose & Cook (132) many years ago

that there is a direct relationship between dietary histidine and urinary allantoin in the rat.

Antifolic acid studies.—A number of chapters in the book "Antimetabolites and Cancer" [Rhoads (133)] contain material relating to antifolic acid compounds. Some of the work presented is original while other parts are reviews of previously published papers. Law (134) has discussed the mechanism of resistance to antifolic acid drugs in a review on the effect of the drugs on various tumors.

The liver glutathione levels of lymphatic leukemia-bearing mice which were treated with amethopterin were assayed by Shacter & Law (135) who found a consistent effect of the antifolic acid compound. However, the effect appeared to result from a demand of the rapidly growing tumor which lowered the liver glutathione level. When the tumor growth was inhibited by the antifolic acid compound the demand lessened and the liver glutathione levels increased.

Webb & Nickerson (136) have reported on the effects of folic acid analogues on growth, cell division, and DNA synthesis in *Candida tropicalis*, *E. coli* and *Bacillus megaterium*. Differential reversal of the inhibition brought about by aminopterin or 4-aminopteroylaspartic acid was obtained with folic acid, thymine, or certain amino acids. Decreases in the DNA content of *E. coli*, grown with aminopterin, dichloraminopterin, or with 4-aminopteroylaspartic acid, were observed. *C. tropicalis* cleaves aminopterin with the liberation of a diazotizable amine residue, according to Nickerson & Webb (137), and thus recovers from initial inhibition.

The activity of a number of xanthones and thiaxanthines as possible interfering agents in the utilization of folic acid was tested by Sobell & Arnold (138). Several members of each of these groups were found to be toxic to *S. faecalis*. The toxic effects of one, 6-chloro-1-{2-[ethyl (2-hydroxypropyl)amino]-ethyl-amino}-4-methylthiaxanthine, could be abolished with thymidine but not with thymine. In no other cases was it possible to link the toxicities to interference with nucleic acid metabolism through finding a relationship with folic acid. Arnold & Sobell (139) have also conducted similar studies with a series of quinoline derivatives. Ten of the 93 compounds were toxic in the presence of limiting amounts of folic acid in the medium. The activity of three of the ten could be reversed, one by CF, one by thymine and one by thymidine. The other seven were too weakly active to warrant further study. In no case did the toxicity anywhere nearly approach that of such compounds as aminopterin or amethopterin.

Interesting differences between the metabolic behavior of the small intestine of the rat and the guinea pig following four days of aminopterin administration were reported by Vitale *et al.* (140). The oxygen uptake of rat intestines was markedly lowered by the treatment, as was the CF content. On the other hand, neither the O_2 uptake nor the CF content of guinea pig small intestine was significantly affected. The normal rat intestine appears

to be in a more rapid state of turnover than the guinea pig intestine, as shown by data on P^{32} uptake.

Passonneau & Totter (141) have reported on the effect of aminopterin on incorporation of formate-C^{14} into DNA and RNA by chick embryos in early stages of growth. Serine in the acid-soluble fraction was also examined. The fixation of glycine-2-C^{14} and adenine-8-C^{14} into RNA and DNA was not inhibited by the antifolic acid compound while the incorporation of formate was markedly diminished.

Broquist (141a) has recently shown that a compound, apparently identical with formiminoglutamic acid, appears in the urine of acute leukemia patients treated with antifolic compounds. The substance was not found in the urine of control patients.

CHOLINE

In a series of papers, Wells and Wells & Mallov have published the results of numerous studies on several choline analogues. In microbiological studies (142), ethanolamine, β-methylcholine and α-methyl-α-hydroxymethylcholine showed anti-choline activity in *Neurospora crassa*. Methyldiethylhomocholine, 1-diethylaminopropanol-2 and α, α-dimethylcholine had anticholine activity for *Saccharomyces carlsbergensis*. Several of the compounds possessed choline-like activity for both organisms. Choline synthesis in young rats was inhibited by feeding 2-amino-2-methyl-propanol-1 (143). The ability of methionine, betaine, and dimethylpropiothetin to prevent choline-deficiency lesions in the treated animals was assayed. The requirement for these compounds was greater than the stoichiometric equivalent of the amount of choline required. These results were interpreted to indicate that the actual synthesis of choline was inhibited by the analogue. When several of the compounds were examined for choline-like activity with segments of rabbit ileum, triethylcholine, β-methyltriethylcholine, and α-methyl-α-hydroxymethylcholine were found to be competitive inhibitors of choline. However, the same compounds showed choline-like activity with segments of jejunum [Wells & Mallov (144)].

It has been known for some time that dimethylaminoethanol is not an active methyl donor. The question as to its fate in metabolism has been answered in part by the work of Johnston & Mackenzie (145) who have shown that this compound gives rise to formaldehyde when incubated with rat liver homogenates. In later work, Mackenzie & Abeles (146) found that the formaldehyde from sarcosine oxidation is first formed as an "active formaldehyde." Presumbably this is identical with, or similar to, other one-carbon fragments in combination with tetrahydrofolic acid. In these experiments, serine was rapidly formed from the glycine, which had been oxidatively demethylated, and the "active formaldehyde."

Glycerophosphorylcholine and phosphorylcholine are again attracting

considerable attention. Dawson has published researches on liver glycerophosphorylcholine diesterase which catalyzes the reaction:

$$\text{L-}\alpha\text{-glycerophosphorylcholine} + H_2O \rightarrow \alpha\text{-glycerophosphoric acid} + \text{choline}$$

Some characteristics of the enzyme were given as well as a method for preparation of the substrate (147). Isotopic experiments indicated that phosphorylcholine was not the sole immediate precursor of liver lecithin but that it probably did play an important part in the synthesis. Insulin hypoglycemia caused a marked rise in liver phosphorylcholine as well as glycerophosphorylcholine (148). Diament (149) did not find free phosphorylcholine in sheep liver, presumably because of the absence of the glycerophosphorylcholine diesterase (147).

Phosphorylcholine and other related N-containing phosphoryl derivatives are hydrolyzed only very slowly by various acid phosphatases, according to Strickland, Thompson & Webster (149a). However, the alkaline phosphatase of brain very rapidly splits this group of compounds.

Kennedy has published detailed methods for the synthesis of several purine and pyrimidine diphosphate cholines (150) and Kennedy & Weiss have studied these compounds as precursors of lecithin (151). Enzymes were found to be widely distributed which carry out the synthesis: cytidine triphosphate + phosphorylcholine → cytidine diphosphate choline + pyrophosphate. Separate enzymes carry out the conversion of CDP choline to lecithin.

Maizel, Benson & Tolbert (152) have identified one of the phosphorus-containing unknowns of Tolbert & Wiebe (153) as phosphorylcholine, a quantitatively important constituent of plant sap. They have discussed the possibility that this compound is a precursor of plant phospholipids and functions also as a transport and storage form of phosphate and nitrogen. DeHeus has published the results of an extensive investigation on the metabolism of choline by *Aspergillus niger* together with a brief review of the metabolism of choline in animals (154). The water-soluble bound form of choline present in the mollusc *Patella vulgata* was identified by Etienne (155) as glycerophosphorylcholine.

Uziel & Hanahan (156) prepared an enzyme from extracts of *Penicillium notatum* which acts on unsaturated lysolecithins to produce in good yields L-α-glycerophosphorylcholine.

Transmethylation.—Verly (157) has reviewed the status of labile methyl metabolism and given the results of some original studies on methanol utilization. It was found that both folic acid and vitamin B_{12} augment the conversion of methanol to choline methyl. Both vitamins were required simultaneously. Also of special interest is his discussion of isotopic effects studied with H, T, D, and C^{14}.

The results of earlier experiments on intermolecular methyl group labeling have been confirmed by du Vigneaud, Rachele & White (158) who used methionine labeled with both C^{14} and D in the same molecules. After three days of feeding the methyl C^{14}D-methionine to rats, also receiving adequate

vitamin B_{12} the ratios of isotopes in the methyl groups of isolated creatine or choline were not significantly different from that of the administered compound.

The influence of dietary gelatin on lipotropic activity of methionine was investigated by Harper & Benton (159). The greater fatty infiltration seen in livers of rats with gelatin replacing part of the casein in the diet was attributed to low threonine and high arginine and glycine. Maw (160) has studied thetin-homocysteine transmethylase in rat liver by estimation of the methionine which is formed in the reaction. The enzyme was partially purified by ammonium sulfate fractionation.

Although the ability of calf liver preparations to catalyze transmethylation reactions was low as compared with rat liver, Hopper & Johnson (161) did not find a specific requirement for choline in calves if 1.2 per cent DL-methionine were present in the diet. The low choline oxidase which is found may account for this ability of the calf to keep pace with its need for choline.

The utilization of methionine for synthesis of choline in rats and in tumor-bearing mice was studied by Stekol *et al.* (162). They concluded that folic acid was not required for the transmethylation but that dimethylaminoethanol was a specific substrate in the transmethylation reactions studied.

The N-methyl group of nicotine as well as the O-methyl groups of lignin may be derived from glycolic acid, according to Byerrum *et al.* (163). Formate did not appear to be an intermediate in the pathway of incorporation of the glycolate α carbon into the methyl groups.

Tcherkes & Filchagin (164) have found that rats on low protein diets excrete larger than normal amounts of N-methylnicotinamide. Addition of dietary methionine or cystine caused a drop in the excretion. This effect was interpreted as a demand for DPN to metabolize the amino acids, hence a lessened tendency for metabolic disposal of the DPN in the presence of the supplementary compounds. In studies on the fate of C^{14} methyl groups attached to the N of amino acids, Nagamatsu (165) found that N-methylphenylalanine was rapidly converted to CO_2, in part. About 47 per cent of the C^{14} of the methyl group was completely oxidized, while about 14 per cent was found in the methyl groups of creatine, choline, and methionine.

Other relationships.—Choline and methionine were found by Davis, Elliot & Lassiter (166) to increase the absorption of carotene fed to young calves. On the other hand, lecithin, choline, or methionine did not influence plasma levels of vitamin A under similar conditions. Dietary choline did not affect absorption of cholesterol by rats, according to Rice, Hungerford Marx (167). Tidwell (168) found, however, that fat supplemented with choline was more rapidly absorbed. At the same time, hydrotropic substances were found to be ineffective in altering the rate of the absorption. Thus the choline did not appear to act by aiding emulsification of the fat. Methionine had little influence while ethionine promoted fat absorption.

The effect of growth hormone and of testosterone on borderline choline-

deficiency states in rats was studied by Wilgram, Best & Blumenstein (169). Both treatments precipitate kidney lesions and initiate cardiovascular changes. A causal relationship between one lesion and the other was not demonstrated, however. DiLuzio & Zilversmit (170) investigated the changes in phospholipid synthesis following choline administration to rabbits maintained on a high fat, low protein, low choline diet. Under these conditions a single dose of intravenous choline further increased the already high rate of liver phosphatide synthesis. Chronic administration of choline depressed the synthesis. The aortic synthesis of phosphatides remained unchanged after the intravenous choline. When high plasma and liver phosphatides were induced by cholesterol feeding no change was found on the rate of incorporation of P^{32} into the phospholipids following choline injection.

New modifications of the periodide method for determining choline have appeared. Both the modifications described by Webster (171) and that of Kushner (172) apply to microgram quantities.

p-Aminobenzoic acid.—Very few publications have appeared on nutritional or vitamin aspects of PABA during the past year. Reid, Martin & Briggs found that folic acid could be spared by PABA in the guinea pig (105). In such circumstances this substance may be thought of as having vitamin action similar to that of carotene. It is possible that PABA may eventually prove either to substitute for or to spare folic acid in other species. Davison *et al.* (173) have investigated the metabolism of radioactive PABA in an *E. coli* mutant which requires it for growth. Four radioactive substances were separated by column chromatography. Two of these showed CF activity but were not identical with CF, being much more labile.

According to Durham (174), the bacterial oxidation of PABA by *Pseudomonas fluorescens* proceeds through the sequence PABA→*p*-hydroxybenzoic acid→protocathechuic acid→*β*-ketoadipic acid.

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WATER-SOLUBLE VITAMINS, PART II[1,2] (BIOTIN, PYRIDOXIN GROUP, NICOTINAMIDE, ASCORBIC ACID)

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Pyridoxin Group

Methods and chemistry.—A notable achievement reported during the past year has been the development by Fujita and co-workers (1–4) of differential methods for the separation and determination of pyridoxine, pyridoxamine, pyridoxal and 4-pyridoxic acid by fluorometric measurements. The principle of the method for the estimation of pyridoxine (1) consists in the removal of interfering substances by permutit, conversion of the vitamin to 4-pyridoxic acid by permanganate oxidation in acid medium, and subsequent lactonization of the oxidation product by heating in acid solution. The lactone can then readily be measured fluorometrically. Pyridoxamine (2) is quantitatively deaminated with nitrite under specified conditions to yield pyridoxine which is assayed as described above. For the analysis of pyridoxal (3), 4-pyridoxic acid is first removed by adsorption on an anionic exchanger, Amberlite IRA-410, and the pyridoxal which passes through the column unadsorbed is then adsorbed on an acidic cationic exchanger, Amberlite IR-112. The 4-pyridoxic acid is eluted from the Amberlite IRA-410 by boiling KCl-acetic acid and is determined as the lactone. Pyridoxal eluted from the IR-112 with *N* NaOH is converted quantitatively to 4-pyridoxic acid by oxidation with ammoniacal silver solution and determined as the lactone. Although the procedure for the fractional determination of the various vitamin B_6 components is somewhat lengthy, it appears to have good specificity; and the values reported for the vitamin B_6 components of animal and vegetable materials show rather good agreement with microbio- and bio-assay data reported in the literature. Because of the preponderance of 4-pyridoxic acid in urine, the differential analysis of this fluid (4) requires a preliminary removal of the acid or its lactone. The 4-pyridoxic acid method of Fujita *et al.* should be a welcome addition to our armamentarium for the study of the metabolism of vitamin B_6 in humans, since the commonly used

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WATER-SOLUBLE VITAMINS, PART II[1,2] (BIOTIN, PYRIDOXIN GROUP, NICOTINAMIDE, ASCORBIC ACID)

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method of Huff & Perlzweig (5) is known to yield unsatisfactory results on urines of subjects unsupplemented with pyridoxine or subsisting on low intakes of vitamin B_6, or when certain interfering substances are present (6–8).

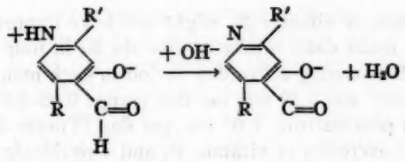
The method has been applied to the determination of the components of blood in three subjects (4) and it is interesting to observe that the total (ave. 2.9 mg./100 ml.) vitamin B_6 active compounds fall within the range (about 2–4 mg./100 ml.) which our laboratory [Marsh *et al.* (9)] has observed in the blood of humans unsupplemented with extra-dietary vitamin B_6. Pyridoxine is absent, as might be expected, and figures for pyridoxamine, pyridoxal and 4-pyridoxic acid are given in terms of the hydrochlorides in the approximate ratio of 18:5:11. This would seem to be the first demonstration of the presence of 4-pyridoxic acid in blood. Naturally, it is doubtful whether the components would be present in native, untreated blood in such a ratio, since there is a good possibility of non-enzymic transamination of the pyridoxal to pyridoxamine during the processing of blood. Also, it is not unlikely that oxidation of pyridoxal to 4-pyridoxic acid might have taken place during the heating which is required for extraction.

Hall *et al.* (10) have attributed the poor reproducibility of the 2,6 dichloroquinonechloroimide reaction for vitamin B_6 at low concentrations to the unstable color of the reaction product. For increased stability of the latter they recommend the use of a 2-phase solvent system: butanol and an aqueous buffer consisting of NH_4OH, NH_4Cl and sodium acetate.

Another paper chromatographic procedure for the separation and quantitative estimation of the free and phosphorylated derivatives of vitamin B_6 is described [Fasella & Baglioni (11)]. The chromatogram is developed in a mixture of propanol and 10 per cent formic acid (80:20) and the components are identified under ultraviolet light either by the color of the fluorescence or by the absorption of light after exposure to NH_3. The spots are eluted with 0.1*N* HCl and the individual components are measured spectrophotometrically. Good separation of the individual components takes place, and the recoveries are satisfactory in all instances with the exception of that for pyridoxal phosphate, which is low (80 per cent).

Bamann & Trapmann (12) have extended their studies on the dephosphorylation of coenzymes by metal ions to riboflavin phosphoric acid and to pyridoxal-3- and 5-phosphoric acids. Pyridoxal-5-phosphate was catalytically dephosphorylated more slowly than the 3-ester by certain rare earth metals (Ce^{+++}, Ce^{++++}, La^{+++}).

Chelation between metals and pyridoxal.—Christensen (13) has presented both spectrophotometric and titrimetric evidence for the differential chelation of alkali metals with pyridoxal. The optical densities of pyridoxal in the presence of metals decreased in the order of Mg, Ca, Li, Na and K, and the amounts of alkali required to produce a given pH were also observed to fall in the same order. The reaction with hydroxyl ion alone was represented as follows:



By titrating in 0.15 *N* NaCl, Christensen arrived at a pK of 8.70. The yellow color which is formed has been attributed to a quinoid tautomer. The results are considered to indicate the occurrence of a second reaction which produces a new absorption.

Christensen argues that pyridoxal contains groupings that make chelation with alkali metals possible in either aqueous or lipoid phases and that there probably is produced a neutral molecule which might have the capability of carrying metal ions through cellular boundaries. Further evidence offered in support of this thesis is the fact that metals which chelate more strongly with pyridoxal, such as Mg^{++}, Li^{+}, and Na^{+}, are the ones that tend to cause the apotryptophanase-pyridoxal phosphate system to dissociate while K^{+} and Rb^{+} tend to stabilize the holoenzyme.

Evidence is also offered (14) for amino acid transfer into cells based upon chelation and Schiff's base formation. The inquiry was undertaken in order to offer an explanation of the observation that pyridoxal enhances the concentration of amino acids by Ehrlich ascites tumor cells of adequately fed mice, whereas cells from vitamin B_6 deficient mice show a deficient concentrating ability.

In an attempt to explain the ability of cells to accumulate diaminopropionic and α-γ-diaminobutyric acids, Christensen & Collins (15) have also investigated metal chelated derivatives of glycine and α-γ-diaminobutyric acid. Chelates of several metals with the Schiff's bases of pyridoxal with glycine and with α-γ-diaminobutyric acid were prepared in the solid state. The latter formed two series of chelates, one representing a monopyridoxylidene and the other a *bis*(pyridoxylidene) derivative. Chelates of pyridoxylidene-glycine and divalent metals appeared to be neutral molecules. The chelates of mono-pyridoxylidene-diaminobutyric acid with Ni and Mn were described as containing 1 metal atom for 2 Schiff base residues, but the corresponding glycine chelates contained a metal ion for each Schiff base residue.

Human nutrition & metabolism.—On intakes of vitamin B_6 averaging 0.86 mg. per day (range 0.52–1.2 mg.) in self-selected diets, the total elimination of vitamin B_6 and metabolites in a small group of nonpregnant women was from three to four times the intake [Marquez & Reynolds (8)]. Seventy-five to eighty-five per cent of the vitamin B_6 was eliminated as 4-pyridoxic acid. During two-thirds of the 15 five day periods of study, the daily intake was less than 1 mg. per day, and the subjects showed a marked loss of nitrogen in nine of the 15 periods. The authors bring up the question whether

more liberal intakes of vitamin B_6 might not have improved the retention of nitrogen. The mean daily intake of vitamin B_6 in four pregnant women on self-selected diets during a five day period in each month of the last trimester of pregnancy was 1.39 mg. per day (range 0.68–2.0 mg.) and during a five day period post-partum, 1.06 mg. per day [Turner & Reynolds (16)]. The total urinary excretion of vitamin B_6 and 4-pyridoxic acid was slightly greater than during a "normal" period of the same women, and was from two to four times the intake, with over 90 per cent of the total representing 4-pyridoxic acid. The authors suggest that this excess excretion over intake furnishes further evidence of synthesis of the vitamin in the body. If this implies synthesis by the intestinal flora, for which there is some evidence, the statement would be acceptable to most investigators. The results which were obtained for the excretion of pyridoxic acid do not support the observations of Beaton & co-workers (17) that less of this metabolite is excreted by pregnant than by non-pregnant women.

High dosages of methionine [Marquez *et al.* (18)] had no effect on the urinary excretion of vitamin B_6 and its metabolites nor on the excretion of N_1-methyl-nicotinamide, but concurrent administration of pyridoxine resulted in increased excretion of N_1-methyl-nicotinamide. This is offered as additional evidence in support of the role of B_6 in the methylation of nicotinamide.

Constant excretion of kynurenine, kynurenic and xanthurenic acid and an inconstant excretion of 3-hydroxykynurenine, 3-hydroxyanthranilic acid and of kynurenine occurred in 10 children subjected to oral tryptophan load tests of 100 mg./kg. body weight over a three day period [Peressini (19)]. Administration of 100 mg. of pyridoxine per day for three days prior to the tryptophan feeding resulted in a constant decrease in the kynurenine, disappearance of xanthurenic acid and a reduction in the conjugates of anthranilic acid and of kynurenine. The results merely suggest that the intake of vitamin B_6 in the children might have been suboptimal.

The report of a human patient with abnormal hematological manifestations which were unaffected by the customary hematopoietic agents but which did respond to intramuscular injections of pyridoxine is of considerable interest [Harris *et al.* (20)]. The anemia was characterized by hypochromia, leukocytosis, elevated serum Fe and an elevated "Fe-binding protein saturation." The appearance of abnormal metabolites in the urine following tryptophan load tests is indicative of an alteration in tryptophan metabolism and is suggestive of either an inadequate intake of vitamin B_6, or some derangement in pyridoxine metabolism.

A more detailed report on the extent, occurrence, and the clinical manifestations of the 1952–1954 outbreak of convulsions in infants ingesting a commercial infant formula low in vitamin B_6 has appeared [Nelson (21)]. Clinical and encephalographic changes associated with seizures as a result of low vitamin B_6 intake in infancy respond rather dramatically to pyridoxine therapy [Coursin (22)]. On this basis it has been suggested that

vitamin B_6 plays an important role in central nervous system metabolism. The metabolism of vitamin B_6 in humans has been reviewed by Vilter (23).

Wachstein & Lobel (24) investigated the urines of 6350 patients (consecutive hospital admissions); 3-hydroxykynurenine was detected by the diazo reaction in the urine of only 11 of these patients. With tryptophan loading, "normal persons" (controls) excreted a number of urinary metabolites, occasionally traces of 3-hydroxykynurenine, and the excretion of xanthurenic acid averaged 24 mg. per 24 hours. In 44 patients with various diseases, the excretion pattern following tryptophan load tests was similar in 28 subjects to that of the control group. The diazo reactions were negative and 3-hydroxykynurenine was not detected in the urine. The xanthurenic acid excretion in the remaining 16 patients averaged 40 mg. per 24 hours; the diazo reactions were positive and the excretion pattern was essentially as described in pregnant women (25). Paper chromatography of these urines revealed the presence of free and acetylated 3-hydroxykynurenine. Repetition of the load tests on a portion of these patients after administration of 50 to 100 mg. of pyridoxine HCl returned the excretion pattern to normal. The authors believe that "the availability of vitamin B_6 is the limiting factor for orderly catabolism of tryptophan under the stress of tryptophan-load tests, even in apparently normal persons." In certain diseases, such as hyperthyroidism, febrile and neoplastic diseases, a smaller amount of vitamin B_6 is available and, as a consequence, metabolites of tryptophan appear in the urine in increased amounts.

The glutamic-aspartic transaminase activity [Glendening *et al.* (26)] of whole blood was observed to be essentially the same in both pregnant and non-pregnant subjects, and the activity was increased significantly by supplementation of the diet with 10 mg. of pyridoxine daily. Transaminase activity of fetal blood was twice as great as that of maternal blood of mothers not receiving any extra-dietary pyridoxine. The transaminase activity of placental tissue was unaffected by prior supplementation of pyridoxine for several weeks. On the basis of these studies the suggestion has been made that "fetal tissues contain optimal quantities of B_6, whereas adults, both pregnant and non-pregnant, contain suboptimal quantities for peak enzymatic activity."

To obtain evidence of pyridoxine deficiency in 17 boys under four years of age with various diseases (hypochromic, microcytic anemia; convulsions, and miscellaneous infectious diseases), Rabe & Plonko (27) measured xanthurenic acid and N_1-methylnicotinamide excretion following standard load tests with DL-tryptophan, and 4-pyridoxic acid and xanthurenic acid excretion before and after parenteral pyridoxine in conjunction with the standard tryptophan load test. No consistent correlation was found between laboratory evidence of pyridoxine depletion and clinical symptoms. The authors concluded on the basis of their own observations and on those in the literature that the laboratory tests may reveal evidence for pyridoxine deficiency in the following order of decreasing sensitivity: xanthurenic acid

excretion, N_1-methylnicotinamide excretion and 4-pyridoxic acid excretion.

Animal nutrition and metabolism.—The activity of betaine-homocysteine transmethylase of the livers of rats fed a diet deficient in one of the following vitamins: thiamine, riboflavin, niacin, pyridoxine, pantothenic acid or folic acid was higher than that of the livers of control animals fed a complete ration [Ericson & Harper (28)]. On the other hand, deficiencies of biotin or choline led to slight decreases in the activity of this enzyme.

Cardiac, renal and hepatic hypertrophy is claimed to occur in rats after a period of 55 days of pyridoxine deficiency [Agnew (29)]. Further work on water metabolism in vitamin deficient rats confirms the fact that the excretion of a combined load of water and inulin is significantly retarded in rats suffering from a deficiency of either pantothenic acid, pyridoxine or riboflavin [Guggenheim (30)]. On the basis of the fact that ACTH or cortisone increased the excretion of both inulin and water (in contrast to control rate), the depressed glomerular filtration has been attributed to adrenal insufficiency.

Evidence for a vitamin B_6 requirement for normal hematopoiesis in the rat has been presented [Dinning & Day (31)]. Rats subjected to severe vitamin B_6 deficiency develop hypochromic anemia with increased erythrocyte count, and a decrease in hemoglobin, mean cell volume, and mean cell hemoglobin which is associated with lymphopenia and granulocytosis. Wertman *et al.* (32) have also observed microcytosis, lymphopenia and granulocytosis in vitamin B_6-deficient rats. In addition, deficient rats exhibited more marked relative granulocytosis and lymphopenia of the bone marrow than the corresponding changes in inanition controls. More fundamental work has elucidated in part the mechanism by which vitamin B_6 plays its role in hematopoiesis. Schulman *et al.* (33) have demonstrated in *in vitro* experiments that pyridoxal phosphate will restore the capacity of red blood cells of vitamin B_6-deficient ducklings to synthesize heme at a normal rate from glycine-2-C^{14}. The addition of pyridoxal phosphate also accelerates the conversion of glycine-2-C^{14} to $C^{14}O_2$ in both normal and deficient erythrocytes. The effect is not dependent on the intact red cell since heme synthesis in hemolysates is also stimulated by pyridoxal phosphate. Results with sodium succinate -2,3-C^{14} were similar to those observed with glycine, but the incorporation of γ-aminolevulinic acid into heme was unaffected by vitamin B_6 deficiency.

Hsu & co-workers (34) report evidence which suggests that there is an impairment of absorption of Co^{60}-labeled-vitamin B_{12} in vitamin B_6-deficient adult female rats. Increased radioactivity appeared in the feces, and decreased radioactivity in the livers and kidneys of the vitamin B_6-deficient animals. Absorption of vitamin B_{12} was unaffected by deficiencies of thiamine, riboflavin and pantothenic acid.

In some very fundamental work, pyridoxal, nicotinamide, choline, pantothenate, riboflavin and vitamin B_1 were found necessary for the growth of Strain L mouse fibroblast cells and Hela cells in tissue culture

[Eagle (35)]. The maximum effective concentrations of the vitamins fell in the range of 10^{-7} to 10^{-8} gm./ml. Flavin-mononucleotide, DPN and thiamine diphosphate (36) have essentially the same activity as the corresponding free vitamins for growth of the mouse fibroblast. A number of coenzymes, flavin-adeninedinucleotide, TPN, coenzyme A and pyridoxal phosphate were significantly less active than the related free vitamins. Pyridoxine and pyridoxal did not differ in their activity, but nicotinic acid was less active than either nicotinamide or DPN in terms of the quantity of growth, even though the effective concentrations exhibited the same order of magnitude. PAB was completely ineffective as a substitute for folic acid. The increased urinary excretion of vitamins of the pyridoxin group by rats subjected to x-irradiation is attributed to the irradiation-induced anorexia and not to the tissue changes resulting from irradiation [Song *et al.* (37)].

The activities of the triacetate, tripalmitate and trilinoleate esters of pyridoxine were equal to that of pyridoxine HCl in the rat, but the fatty acid esters appeared to be retained in the body for a longer time than pyridoxal, pyridoxamine and pyridoxine [Sakuragi & Kummerow (38)]. Some evidence suggested that at least a part of the fatty acid ester could be stored in the body without prior complete hydrolysis. The tribenzoate, tri-*p*-nitro- and tri-*m*-nitrobenzoate esters of pyridoxine possessed biological activity, but the potencies of the *p*- and *m*-nitrobenzoates were extremely low.

The normal rat secretes cholic acid conjugated with taurine, but in bile fistula rats maintained on a diet deficient in cystine and methionine and subjected to severe vitamin B_6 deficiency, the amount of taurocholic acid is decreased and an increased amount of glycocholic acid appears in the bile [Doisy *et al.* (39)]. Repletion of the deficient rats with a complete ration results in the disappearance of the abnormal conjugate (glycocholic aid) and a reappearance of taurocholic acid in the bile. The mechanism for this abnormality is quite likely related to the fact that pyridoxal phosphate is the coenzyme for the decarboxylation of cysteinesulfinic acid (40) and hence under the conditions of the experiment one would expect a block in the formation of taurine.

Studies on essential fatty acid and vitamin B_6 deficiency in rats [Tulpule & Williams (41)] again demonstrate that vitamin B_6 deficiency accentuates the effects of an EFA deficiency. In EFA deficiency there is a marked decrease in the ratio of phosphate esterified to O_2 consumed for the oxidation of both reduced DPN and reduced cytochrome c. On this basis it has been suggested that vitamin B_6 is required for the utilization of linoleate in "maintaining the phosphate-esterification system associated with the oxidation of reduced cytochrome c." Irrespective of the previous pyridoxine-deficient regimen such as simple pyridoxine deficiency, semi-starvation, a high protein regimen, or a non-protein regimen, the rat is able to replete its tissue pyridoxine concentrations to normal when fed a complete, 20 per cent casein ration ad libitum for two weeks [Tulpule & Williams (42)].

The vitamin B_6-deficient rhesus monkey represents a veritable museum of pathology. Neuropathologic changes of the central nervous system have been ascribed to a deficiency of this vitamin [Victor & Adams (43)]. In addition to arteriosclerotic lesions, an increased incidence of dental caries and the development of fatty metamorphosis or cirrhosis of the liver have been reported [Rinehart & Greenberg (44)]. The same authors have estimated the vitamin B_6 requirement for growth to be 62 μg. of pyridoxine HCl per kilogram body weight per day, a value which is in accord with values previously reported for the pig (45) and for the dog (46). Nutritional muscular dystrophy has been induced in the monkey on a diet deficient in tocopherol and vitamin B_6 [Day & Dinning (47)]. Since all manifestations of the disease responded to the administration of α-tocopherol alone, the part that vitamin B_6 plays in the development of the syndrome remains obscure. A review of the evidence for the role of pyridoxine in neuronal metabolism by Tower (48) has led to the suggestion that deficiency of pyridoxine is associated with disturbances in the metabolism of the neural lipides of myelin structures.

Rabbits maintained on a vitamin B_6-deficient diet exhibited the following manifestations of deficiency: dermatitis, anemia, xanthurenic acid excretion and an increase in blood clotting time. Neurological manifestations consisting of trembling, incoordination and convulsions were also described [Hove & Herndon (49)].

Micro-organisms.—More direct evidence for the role of vitamin B_6 in the synthesis of proteins is provided by Lichstein (50), who observed that the synthesis of the apoenzyme of a strain of *Escherichia coli* is dependent on the presence of the vitamin. This adds support to earlier work by Axelrod (51) who reported that the low antibody titers in vitamin B_6-deficient rats could be increased by vitamin B_6 supplementation.

Proteus vulgaris contains a single enzyme system requiring pyridoxal phosphate [Ekladius & King (52)] which can decarboxylate both valine and leucine. Evidence is cited in support of the idea that the affinity of the enzyme for the coenzyme may be influenced by the substrate being metabolized. Cell-free extracts decarboxylate valine slowly, and show no activity toward leucine. However, activity towards both substrates is restored completely by the addition of pyridoxal phosphate, but not by pyridoxal. Larger quantities of enzyme are required for the decarboxylation of leucine, the K_s values (half-maximum velocity) being 0.3×10^{-6} M for valine and 5×10^{-6} M for leucine. This difference in affinity is evident also in the greater sensitivity of leucine decarboxylation to inhibition by carbonyl "fixatives" such as hydroxylamine and semicarbazide.

The addition of certain amino acids to the growth of a wild type of *Neurospora crassa* (mating type A, No. 126–1) has been observed to increase the concentration of certain transaminases in mycelial extracts [Finchain & Boulter (53)]. To explain the observed effects the authors postulate the presence of at least four different enzymes: alanine-glutamate, aspartate-glutamate, ornithine-glutamate, and a fourth enzyme which is capable of

catalyzing glutamate formation from a number of different amino acids (phenylalanine, valine, isoleucine, leucine, norleucine, norvaline, methionine, α-amino-butyric acid and tryptophan). Moreover, alanine-transaminase is judged to be an unstable enzyme which is easily dissociated from its coenzyme, pyridoxal phosphate.

Quantitative reduction of DL-proline to δ-aminovaleric acid is catalyzed by a proline-reductase system of clostridium, strain HF [Stadtman (54)]. A number of dithiols act as reducing agents or as electron donors in the reaction with unfractionated extracts, but are inactive with a purified enzyme. The cofactors required for maximum proline reduction in the isolated sytsem have been determined and consist of: DPN, Mg^{++} and pyridoxal phosphate.

Casein hydrolysate and certain amino acids inhibit the utilization of tryptophan by a nicotinic acid-requiring strain of *N. crassa* [Sundaram *et al.* (55)]. This effect is overcome by thiamine, pyridoxine, folic acid and choline.

An enzyme system which converts anthranilate to indole has been extracted from *E. coli* [Trudinger (56)]. The reaction requires ATP, inorganic phosphorus, pyridoxal phosphate, a carbon donor and Mg^{++} or Mn^{++}. Pyruvate and AKG served as the most effective carbon donors.

Vitamin B_6-enzymes.—Several reports on decarboxylases of both plant and animal origin have appeared. Glutamic acid decarboxylase has been detected in *Rhodotorula glutanis* [Krishnaswamy & Giri (57)]. Pyridoxal phosphate was found to restore the activity of dialyzed preparations of the enzyme and to reverse the inhibition produced by hydroxylamine. The flavedo of lemons and oranges has been described as being an extremely rich source of glutamic decarboxylase [Axelrod *et al.* (58)]. Crude fractionation yields a preparation with an activity of approximately 40,000 cu. mm. of CO_2/hr./mg. of protein N. The fact that the enzyme exhibits a pyridoxal phosphate requirement when incubated at 37° and pH 5.6 and not when the temperature is lowered to 15° or the pH changed to 6.5 would suggest that the enzyme is easily dissociated from its coenzyme. Orange and lemon flavedo would seem to be excellent sources of the enzyme for the determination of glutamic acid. A preparation of decarboxylase from *Mycobacterium phlei* has been adapted to the determination of L-glutamic acid (59).

By the use of purified enzyme preparations it has been shown that thiol in addition to pyridoxal phosphate groups is essential for the activity of the cysteinesulfinic acid decarboxylases of brain and liver [Davison (60)]. Convincing evidence is presented in favor of the idea that these decarboxylases of brain and liver are two different enzymes, and that a single enzyme in brain is capable of decarboxylating glutamic, cysteinesulfinic and cysteic acids. A new enzyme has been observed in the posterior glands of the silk worm and in rat liver homogenates which catalyzes the decarboxylation of aminomalonic acid to glycine [Shimura *et al.* (61)]. Rabbit platelets have been shown to decarboxylate C^{14} L-histidine and to bind the histamine formed in a stable state (62). A soluble preparation of this enzyme has been prepared. Another enzyme, diamine oxidase or histaminase, has been added

to the list of enzymes which require pyridoxal phosphate as coenzyme [Davison (63)].

Previous investigations (64, 65) suggested that pyridoxal phosphate is the coenzyme for 5-hydroxytryptophan decarboxylase. Additional evidence has now been brought forth to support this hypothesis [Buxton & Sinclair (66)]. The amount of 5-hydroxytryptamine formed from 5-hydroxytryptophan during incubation with kidney tissue from rats subjected to a pyridoxine deficient diet and desoxypyridoxine was only about one-fourth that of pair fed controls. The activity of the deficient kidney tissue was restored to near normal values by *in vitro* addition of pyridoxal phosphate.

Resolution of cysteine desulfhydrase of *E. coli* with respect to its coenzyme, pyridoxal phosphate, has been attained by grinding the bacterial cells with alumina [Metaxas & Delwiche (67)]. The resulting enzyme solution exhibited negligible activity in the absence of pyridoxal phosphate and was uninfluenced by adenosine-5′-phosphate and biotin, but was inhibited by AKG, tryptophan, glutamic acid, alanine and tris(hydroxymethyl)-aminomethane. A Michaelis constant of 1.76×10^{-2} M/L was obtained for enzymatic action at pH 7.8 and 40°.

Kynureninase of porcine liver has been enriched 150 fold by a newly described method [Wiss & Weber (68)]. The most active preparations were homogeneous by both electrophoretic and ultracentrifugal analysis. Vitamin B_6 was present in the hydrolysate of kynureninase, and this fact, coupled with the earlier observation that pyridoxal phosphate activates the enzyme, provides adequate evidence that pyridoxal phosphate is the coenzyme for kynureninase. Acylpyruvase has been prepared by the same authors (69) and has been shown to differ from kynureninase. It cleaves acylpyruvate and is not activated by pyridoxal phosphate.

Some new transamination reactions have been described. Transamination between alanine and hydroxypyruvate to form serine and pyruvate has been reported to occur in the liver and kidney of several animals [Sallach (70)]. Partial purification of a dog liver homogenate has been achieved and the resulting enzyme preparation was stimulated by pyridoxal phosphate. Cell-free extracts of the pseudomonad strain 2 RCC-1 are capable of synthesizing glycine from alanine, aspartic acid, glutamic acid, asparagine and glutamine by transamination with glyoxylic acid [Campbell (71)]. These also represent pyridoxal phosphate-dependent reactions. Through the use of INH to produce a more severe pyridoxine deficiency in rats, evidence has been provided for a role of vitamin B_6 in the glutamine-α-ketoacid transamination-deamidation reaction [Meister & Downey (72)].

The use of N^{15}-labeled substrates for the study of the transamination reaction in a pig heart homogenate, has made it possible to ascertain whether an amino donor transaminates to AKG [Tanenbaum (73)]. Alanine, aspartic acid, leucine, valine, phenylalanine, tyrosine, serine, glycine, and lysine can undergo such a transamination. Histidine does not react as an amino donor, and NH_3 does not act as an intermediate in the amino group transfer.

N^{15}-labeled pyridoxamine was observed to transaminate readily with AKG.

Vitamin analogues and antagonism.—The mechanism of action of isonicotinylhydrazide and of its effects in micro-organisms and in animals continues to be studied rather extensively. Several investigations have dealt with the effect of this compound on bacterial and mammalian decarboxylases. Diaminopimelic decarboxylase from *Aerobacter aerogenes* and lysine decarboxylase were inhibited by INH in the absence of substrate, and the degree of inhibition was increased with the period of preincubation with INH [Hoare (74)]. The inhibition of ω-aspartic acid decarboxylase of *Desulfovibrio desulfuricans* by the hydrazide is proportional to the logarithm of the INH concentration [Cattaneo-Lacombe & Senez (75)]. Generally a concentration of 1.25×10^{-3} *M* was required to decrease the activity of the enzyme preparation 50 per cent. Pyridoxal phosphate (10^{-3} *M*) reactivated enzyme preparations which either had been inactivated by prior, prolonged dialysis or inhibited by INH. Of further interest is the fact that extremely small concentrations of AKG increase the liberation of CO_2 from ω-aspartic acid by the enzyme. The effect of the AKG has been attributed to a reactivation of pyridoxal phosphate by the former and not to any intermediate transamination (76). Moreover, AKG also reactivates INH-inhibited enzyme extracts in concentrations which are 1000 times lower than that of the inhibitor, a fact which would seem to preclude simple combination of AKG and INH as explanation of the reactivating effect of AKG. Of a large number of metabolites studied, pyridoxal was the most effective antagonist against the inhibitory effects of INH on *M. tuberculosis* H 37 RV [Pope (77)]. Other compounds which had a similar effect included pyridoxamine, sodium pyruvate, AKG, vitamin B_{12}, guanine, nucleic acid and a number of amino acids, obviously a very non-specific type of antagonism. Tubercle bacilli which were resistant to 10 μg. of INH per ml. were observed to be strongly inhibited by the drug when exposed to sub-inhibitory concentrations of pyridoxal. Endogenous respiration of tubercle bacilli was inhibited by high concentrations of INH, and could be counteracted by pyridoxal or by AKG.

Vilter *et al.* (78) suggested that the increased excretion of Vitamin B_6 activity in the urine of humans following INH treatment was the result of the formation and excretion of a hydrazone of INH. Since Williams and associates (79) were able to detect pyridoxal-semicarbazone in the urine of semicarbazide-treated dogs, they concluded that INH and other convulsant drugs combined with pyridoxal to form hydrazones. Rat liver and brain cysteinesulfinic acid and brain glutamic acid decarboxylases were inhibited by INH [Davison (80)]. For this reason he suggested that the inhibition was the result of the formation of a pyridoxal phosphate-INH-hydrazone. Experimental activation energies of 16 and 14 KCal/mole, respectively, for the reaction with INH and pyridoxal phosphate in the presence and absence of vitamin B_6-dependent enzymes were presented in support of the thesis. Wiegand (81) has determined the specific reaction rates and the activation energies for the formation of a number of pyridoxal phosphate hydrazones

from pyridoxal-5-phosphate and a series of hydrazides. The acid dissociation constants of the hydrazones were found to be linearly related to the specific reaction rates, but no correlation could be demonstrated between the convulsant potency of the hydrazides and the rate of complex formation with pyridoxal phosphate. Studies with C^{14} labeled INH demonstrated a lowered rate of metabolism and excretion of the drug and its metabolites in the vitamin B_6-deficient rat, but failed to give a complete explanation for its greater toxicity in the pyridoxine-deprived rat [Boone *et al.* (82)].

The parallelism between the INH-neuritis induced in rats by chronic oral administration of INH (200–250 mg./kg./day) and that observed in patients in the clinic is pointed out [Zbinden & Studer (83)]. Degenerative changes occur in the peripheral nerves of the rat which after 7 to 8 months of treatment lead to crippling of the extremities. Histologically, localized demyelination occurs often associated with the breakdown of the axis cylinders and proliferation of the sheaths of Schwann. Simultaneous injection of pyridoxine (50 mg./kg./day) prevents the nerve alterations. The lesions are also inhibited by intramuscular injection of pure pyridoxal phosphate. On a molar basis (84) the latter is approximately equivalent to pyridoxal in counteracting the INH-induced peripheral nerve degeneration. Pyridoxal-isonicotinyl hydrazone, which is 17 times weaker than INH as an antituberculous agent in mouse tuberculosis, results in an almost insignificant degeneration of peripheral nerves.

Differences in the metabolism of tryptophan in INH-treated, as compared to vitamin B_6-deprived rats, are described [Charconnet-Harding *et al.* (85)]. Administration of L-tryptophan to INH-treated rats did not increase xanthurenic acid excretion as it does in vitamin B_6 deficiency. Derivatives of kynurenine were also absent or present in only trace amounts in the urines of the former. D-tryptophan did not affect the excretion of xanthurenic acid in either condition, but the excretion of indole derivatives was markedly increased in both. Blocking of the conversion of D- to L-tryptophan by INH is presented as an explanation for the latter.

Semicarbazide, thiosemicarbazide, and thiocarbohydrazide, given to schizophrenic patients orally, induce temporary epileptic states for one to five hours [Pfeiffer *et al.* (86)]. Under these circumstances, seizures which may occur spontaneously or which may be induced by auditory or photic stimulation, can be antidoted completely by pyridoxine. Although no data are presented, the authors report that work in progress indicates significant reduction in tissue pyridoxine levels following hydrazide administration.

In neurospora the biosynthesis of thiamine is inhibited by pyridoxine or by deoxypyridoxine [Harris (87)]. The inhibition is antagonized noncompetitively by thiazole. Because of the structural similarity of pyridoxine and deoxypyridoxine to pyrimidine, the suggestion has been made that pyridoxine prevents the utilization of pyrimidine in a competitive manner.

Chick fibroblasts perfused with varying concentrations of deoxypyridoxine in Tyrode's solution manifested nuclear and cytoplasmic changes

in 48 to 96 hours [Gillette (88)]. The relative number of nucleolar bodies increased and the nucleus itself became elongated. The movement in the mitochondrial elements within the cells progressively slowed until no apparent activity was evident in the cytoplasm.

Hurwitz [89] has extended his studies on the enzymic phosphorylation of vitamin B_6 analogues, and has investigated their effect upon tyrosine decarboxylase. The kinase preparation which phosphorylated pyridoxal in the presence of ATP also phosphorylated certain analogues but failed to phosphorylate other pyridoxine analogues. The pyridine derivatives which were phosphorylated were unsubstituted in the 6-position and contained the hydroxymethyl group in the 5-position.

Further studies on hydroxymethylpyrimidine (toxopyrimidine) have been reported. Shintani (90) found that the convulsant effect of the compound in mice was counteracted by vitamins of the B_6-group in the following increasing order of effectiveness (the figures in parentheses represent the ratio of anticonvulsant:convulsant): pyridoxamine (1/5), pyridoxine (1/100) and pyridoxal (1/200). The convulsant effect of the pyrimidine was increased in mice on a vitamin B_6-deficient diet and the antagonistic effect of pyridoxal was decreased. Rats which received a diet deficient both in vitamin B_6 and the pyrimidine developed acrodynia and ceased to grow in 2–3 weeks. Urinary excretion of xanthurenic acid following tryptophan feeding was increased by the administration of toxopyrimidine. Growth inhibition of *Saccharomyces carlsbergensis* occurred in the presence of the compound and was antagonized competitively by pyridoxal.

Nicotinic Acid

Nutrition and metabolism.—The metabolism of nicotinic acid in humans is further clarified by studies on the excretion of N-methyl-2-pyridone-5-carboxamide following oral administration of known or potential precursors to male subjects [Walter *et al.* (91)]. The following is the probable sequence of reactions which nicotinic acid undergoes in its conversion to the pyridone derivative: amidation, methylation and oxidation. The free acid, N_1-methyl-2-pyridone-5-carboxylic acid and its glycine conjugate, N_1-methyl-2-pyridone-5-formamidoacetic acid, have been isolated from human urine [Lindenblad *et al.* (92)]. From the same laboratory quantitative studies of the urinary metabolites of D-, DL-, acetyl L- and acetyl-D-tryptophan in the human (93) and a comparative study of the quantitative excretion of the urinary metabolites of the dog, cat, rat and man (94) have been presented.

Following oral administration of a dose of the free acid to a human subject, 30 per cent of the dose was eliminated as equal quantities of the glycine conjugate and the free acid. The relation between urinary excretion of nicotinic acid and its metabolites and dietary intake of nicotinic acid and tryptophan has been examined in a group of young women [Frazier *et al.* (95)].

Large doses of nicotinic acid and its amide in humans produce temporary changes in the leukocytes and alter the excretion of urinary constituents

[Hoffer (96)]. Nicotinic acid decreases the ratio of uric acid:creatinine while the amide increases the ratio. Also, nicotinic acid acidifies the urine and results in increased excretion of Na^+ and a decrease in urinary glycine, but the amide lowers urine acidity and increases the K^+ and Na^+ excretion and results in a marked increase in glycine excretion. Nicotinic acid when administered in large doses is excreted as nicotinuric acid, thus depletes the glycine pool, and results in a decrease in uric acid production. The amide is excreted as N_1-CH_3-nicotinamide and hence does not deplete the glycine pool.

An unusual case of indicanuria in a boy who developed pellagra with marked mental disturbances on a diet presumed to be normal has been described [Rodnight & McIlwain (97)]. Urinary nicotinic acid derivatives were not excreted in extraordinary amounts on an ordinary diet or one supplemented with nicotinic acid. The suggestion is proposed that the mental disorder associated with the indicanuria may be the result of a deviation of tryptophan metabolism. Evidence for the replacement of nicotinamide by INH in DPN in experimental systems exists [Zatman *et al.* (98)], but there has been no demonstration of INH-nicotinamide antagonism in the intact experimental animal. Clinical evidence suggests the possible occurrence of such an antagonism in humans. Harrison and associates (99) describe the dramatic response to niacinamide therapy in a poorly nourished woman who developed classical manifestations of pellagra following concurrent treatment with INH and pyridoxine for disseminated sclerosis. The possibility that the niacin stores of the patient were impoverished is not unlikely, since her diet was inadequate both as to nutritional quality and intake. Other cases which have developed pellagra under similar circumstances have been reported in the literature (100, 101).

From studies on the metabolism of thiamine, riboflavin, and nicotinic acid in schizophrenic patients, Zelin'skii (102) has concluded that there is a derangement in vitamin metabolism in schizophrenia which lowers the process of phosphorylation, and leads to a lack of incorporation of vitamins into the enzyme systems. In addition, the blood levels of DPN and TPN were unaffected by intensive treatment with nicotinic acid. The mean nicotinic acid level of the blood of 53 pellagrins (4.93 ± 1.49) did not differ significantly from that (6.02 ± 1.37) of 15 healthy individuals [Mezincescu *et al.* (103)]. Similarly, Bartlett (104) observed no correlation between the concentration of niacin in the erythrocytes and its intake in 86 children.

Oral administration of 100 mg. of tryptophan or 2.5 mg. of anthranilic acid per day increased the survival period of adrenalectomized rats and prevented a rapid fall in hepatic glycogen [Kotake & Inouye (105)]. In this respect, nicotinic acid, vitamin C, and L-methionine were reported to be much less effective. According to Orunesu *et al.* (106), large doses of nicotinamide induce hepatic glycogenolysis by affecting the activity of glucose-6-phosphate dehydrogenase. The uptake of injected I^{131} by the thyroid and pituitary glands of rabbits was increased by the injection of nicotinic acid

[Siver *et al.* (107)]. The excretion of N_1-methyl-nicotinamide is increased in hypothyroid rats and decreased in hyperthyroid rats following the injection of nicotinamide [Sundaram *et al.* (108)].

In tryptophan-niacin depleted rats, greater supplements of tryptophan were required for the synthesis of blood pyridine nucleotides than for growth, which in turn was greater than the requirement for the maintenance of nitrogen equilibrium. Niacin was ineffective in restoring blood pyridine-nucleotides in the absence of tryptophan, but the combination of the two was most effective [Chaloupka (109)].

Diminished niacin synthesis as a result of an increase of an enzyme which diverts tryptophan metabolism through an alternate pathway (picolinic acid) has been offered as an explanation for the lowered N_1-methyl nicotinamide excretion in alloxan-diabetic rats [Mehler *et al.* (110)]. Ovarectomy in adult female rats resulted in a decreased excretion of N_1-methyl-nicotinamide and acid hydrolyzable nicotinic acid metabolites which were markedly increased by the administration of progesterone and estrone [Lojkin *et al.* (111)]. High levels of sodium nicotinate or niacinamide retarded liver regeneration in partially hepatectomized rats; on the other hand coramine (N_1-N-diethylnicotinamide), by subcutaneous or oral administration, accelerated the rate of liver degeneration [Gershbein (112)]. Liver and kidney tissue of the rat are able to methylate nicotinamide *in vitro* to form two compounds which are presumed to be intermediates in the formation of pyridine coenzymes [Porcellati *et al.* (113)].

The metabolism of nicotinamide in the chick is markedly different from that in mammals [Chang & Johnson (114)]. β-Nicotinyl-D-glucuronic acid, nicotinuric acid, nicotinamide, 5-mono-, α-mono- and 2,5-dinicotinylornithine have been identified as urinary metabolites following the administration of C^{14} labeled nicotinamide. The same metabolites were also present in liver and kidney.

DPN-chemistry and DPN-enzymes.—The oxidoclase system in rat liver which plays a role in the biogenesis of cholesterol by catalyzing the cyclization of squalene to lanosterol requires O_2 and either DPNH or TPNH [Tchen & Bloch (115)]. Ernster [116, 117] has demonstrated reversible uncoupling of oxidative phosphorylation in DPN-linked systems of mitochondria and has presented evidence that DPN and DPNH are reversibly associated with the structure of the mitochondria in such a way that the association of DPNH is firmer than that of DPN. DPN is a cofactor for galactowaldenase [Maxwell (118)]. Inversion does not occur when the purified enzyme is incubated with uridinediphosphogalactose unless DPN is added. DPN and TPN are required for maximum synthesis of higher fatty acids from acetic acid by a soluble dialyzed fraction of rat liver (Langdon [119)]. DPN acts as an electron acceptor of chicken-liver xanthine dehydrogenase, but does not appear to play the same role for milk xanthine oxidase [Morell (120)]. DPNH peroxidase of *Streptococcus faecalis*, a flavoprotein peroxidase which contains flavin-adeninedinucleotide, combines with

DPNH and other reduced pyridine nucleotides to form enzyme-substrate complexes. DPNH combines in such a manner that reduction is blocked [Dolin (121)]. Deuterium exchange occurs with DPN in alkaline solutions at the 2-position of the niacinamide moiety [San Pietro (122)]. A possible mechanism for the exchange is based on ionization of the pyridine ring hydrogen. The mechanism proposed for the reduction of DPN by sodium dithionite proceeds via the formation of a sulfinate at C4 of the pyridine ring, followed by hydrolysis of the sulfinate to yield sulfite and reduced DPN (123). Electrolytically reduced DPN is identical with the enzymically reduced product in absorption spectra and Rf value, but otherwise differs from the latter in not exhibiting fluorescence and in being completely inactive toward alcohol or lactic dehydrogenase [Ke (124)]. Irradiation of TPN and DPN with intense light in the ultraviolet region between 210 and 280 mμ leads to inactivation [Seraydarian (125)].

A sensitive and accurate spectrophotometric method for the estimation of oxidized and reduced DPN and TPN in quantities as small as 0.1 μg has been developed [Glock & McLean (126)]. The examinations of a number of tissues has revealed that DPN occurs primarily in the oxidized and TPN predominantly or exclusively in the reduced form (127). Liver, adrenals, kidney, and lactating mammary gland contain high total concentrations of both coenzymes, while the level of TPN is high in the ovary and low in brain and skeletal muscle. Pyridine nucleotide synthesis has been studied in the mouse, where it has been shown that the concentration is increased in the liver tenfold, primarily as DPN, following injection of nicotinamide, and is elevated in the spleen, kidney and neoplastic tissue [Kaplan *et al.* (128)]. The increase and also the subsequent breakdown of DPN were inhibited by 6-mercaptopurine.

The capacity of homogenates of liver from transplanted tumor bearing mice to synthesize DPN was reduced 30 to 50 per cent [Waravdekar *et al.* (129)]. When sarcoma 37 was removed surgically from the host, the capacity of the liver to synthesize DPN approached that of control mice. Of further interest is the occurrence of a marked reduction in the DPN-synthesizing power of blood within one day after tumor-inoculation of mice. The Diphosphopyridine nucleotidase of the rabbit erythrocyte is bound firmly to the cell membrane, and its distribution is such that it is able to react with DPN in the external medium [Alvisatos *et al.* (130)]. It cleaves nicotinamide from both TPN and DPN but the affinity for TPN is only approximately one-third of that for DPN. It is present also in the rabbit reticulocyte [Rubenstein *et al.* (131)]. DPN pyrophosphorylase activity occurs in the chicken erythrocyte, but could not be detected in rabbit reticulocytes nor in human or rabbit erythrocytes [Malkin *et al.* (132)].

Rapid oxidation of DPNH and TPNH occurs with an aqueous extract of beef brain cortex in the presence of the following electron acceptors: 2-methyl-1,4-napthoquinone, adrenochrome, methylene blue and 2,6 dichlorophenolindophenol. TPNH was oxidized more rapidly in the presence

of the naphthoquinone and DPN in the presence of adrenochrome [Englard & Strecker (133)].

Analogues and antagonism.—The administration of 3-acetylpyridine to rats did not inhibit the formation of pyridine nucleotides from tryptophan and nicotinic acid, but led to an increase in a substance which behaved like DPN [Hayman *et al.* (134)]. Augmentation of the pyridine nucleotides of the liver occurred with and without concurrent administration of nicotinic acid or tryptophan. Since Kaplan *et al.* (135) have demonstrated the formation of acetylpyridine-DPN in an *in vitro* system employing pig brain Diphosphopyridine nucleotidase, it is rather likely that this occurs in the intact animal and offers an explanation for the increase in "apparent" DPN following administration of acetylpyridine.

6-aminonicotinamide was the only member of a group of nicotinic acid analogues tested which inhibited nicotinamidase activity in cell-free extracts of *M. phlei* [Grosswicz & Halpern (136)]. The reduction of DPN by crystalline alcohol dehydrogenase is inhibited by 3- and 4-substituted pyridine compounds and the inhibition is proportional to the pKa of the ring nitrogen [Van Eys (137)].

By using pig brain Diphosphopyridine nucleatidase as a catalyst in the exchange reaction between DPN and the respective niacin antagonists, 6-aminonicotinamide analogue of DPN [Johnson & McColl (138)] and that of 3-acetylpyridine [Kaplan *et al.* (135)] have been prepared and isolated. Both the 6-aminonicotinamide-DPN (138) and the acetylpyridine-DPN (139) have been detected in tissues of the intact mouse following treatment with the antagonists; also both of the unnatural pyridine nucleotides formed cyanide addition compounds. The 6-aminonicotinamide-DPN is inactive in the alcohol dehydrogenase system, whereas the acetylpyridine and also the pyridine-3-aldehyde analogues of DPN were found to be active in a number of dehydrogenase systems [Kaplan *et al.* (140)].

Biotin

Genghof (141) has described the production of four vitamers by *Corynebacterium xerose* (ATTC No. 9016) when grown in the presence of pimelic acid. One of these has been characterized as probably being desthiobiotin. When grown in the presence of suberic and azelaic acids, homologues of pimelic acid, 45 and 100-fold less biotin activity was produced in the culture than with pimelic acid. Tatum (142) had previously shown the production of dethiobiotin by *Penicillium chrysogenum*, and Wright *et al.* (143) of biotin-sulfoxide in media which contained pimelic acid.

Previous theories relating to the reduction of the biotin-requirement in lactic acid bacteria by fatty acids have suggested that biotin may function in some unknown manner in the biosynthesis of oleic and other fatty acids (144), or that other surface active agents could replace biotin, which itself exhibits some degree of surface activity. A recent study [Traub & Lichstein (145)] suggests that cells of this species are relatively impermeable to biotin

and that oleic acid appears to act by permitting more efficient entry of the vitamin into the bacterial cell.

Direct microbiological assays in conjunction with bioautographic procedures have revealed that human urine contains, in addition to rather large amounts of free biotin, very small quantities of a "hydrophilic" biotin derivative which may be "biocytin sulfoxide" [Wright *et al.* (146)]. With the procedures used, the biotin in the urine was observed to be avidin-combinable, and no evidence was obtained for the presence of any of the "unusual" biotin derivatives described by others (147, 148).

X-irradiation, with doses of 100-500 Kr, of *Allescheria boydii* 1699, an ascomycete requiring biotin, in a suspension with and without biotin, resulted in a decreased Qo_2, regardless of the presence or absence of biotin [Morczek & Mucke (149)]. When the dose was increased beyond 400 Kr, a "residual respiration" of approximately 45 per cent of the control values remained and was attributed to the redox potentials of the reaction groups which were formed during the irradiation of the protein of the fungus.

Luckey & associates (150) have studied vitamin metabolism in germ-free rats. In contrast to ordinary rats, the latter were found to require a dietary source of biotin. Biotin appeared to be required for the biosynthesis of folic acid. Vitamin balance studies revealed that less niacin, riboflavin and biotin were excreted, and less accumulated in the body tissues than were fed to these rats.

The incorporation of S^{35} labeled methionine into the proteins of different organs is lower in biotin-deficient than in control rats [Kritsman *et al.* (151)]. The administration of 10 μg of biotin per day on two successive days prior to the injection of the labeled methionine increased its incorporation in the proteins of the biotin-deficient rat to normal. *In vitro* experiments with liver slices and with blood plasma were reported to yield similar results.

Biotin deficiency induced in male rats by feeding a diet which contained egg white results in early signs of testicular degeneration with retarded spermatogenesis and degeneration of spermatocytes [Delost & Terroine (152)]. Testicular lesions were also observed by Katsh *et al.* (153) under similar conditions. The former group suggests that the testicular degeneration may be the result of deficient production of male hormone.

According to Terroine (154) severe biotin deficiency in the rat results in an increase in pyruvate, moderate hypoglycemia, a weakened reaction to glucose, and a marked reduction in the amount of reducing sugar in the liver. The amounts of glycogen and free reducing sugar are unaltered and liver glycogen is changed little. Of considerable interest is a report by Sundaram & Sarma (155) that the biotin-deficient rat is incapable of converting an appreciable amount of added tryptophan to N-methylnicotinamide and nicotinic acid. It is difficult to reconcile this observation with that of Dalgliesh (156), who found that the excretion patterns of kynurenine, hydroxykynurenine, xanthurenic acid, and anthranilic acid and their con-

jugates, following a test dose of tryptophan, were unaltered by biotin deficiency.

Ascorbic Acid

Methods.—A new method for the determination of dehydroascorbic acid has been developed [Hughes (157)], in which the latter is rapidly reduced by homocysteine at pH 7.0 followed by titration with 2:6-dichlorophenolindophenol. Under the conditions specified, excess homocysteine causes no interference. The procedure is claimed to be more rapid and specific than other available methods. Titration of ascorbic acid with 1,2 naphthoquinone-4-sulfonate has been made the basis of a procedure for the estimation of ascorbic acid [Barakat *et al.* (158)]. The naphthoquinone derivative oxidizes the vitamin rapidly and quantitatively to dehydroascorbic acid.

Clinical studies and metabolism in humans.—From an investigation of 33 hospitalized urban Bantu infants suffering from malnutrition and other diseases and 21 "normal" infants, infantile scurvy is described as being rare in the South African Bantu [Andersson *et al.* (159)]. In spite of the fact that foods given to the infants were poor in vitamin C, the plasma ascorbic levels of the infants were stated to be consistent with the "absence of the disease". The authors speculate on the possibility of endogenous production of the vitamin. Since thus far there is no evidence for such synthesis in humans, perhaps it would be desirable to determine if the Bantu has a low requirement for ascorbic acid. According to Grusin & Kincaid-Smith (160) scurvy with manifestations of an unusual chronic leg lesion occurs in the adult South African Bantu. In contrast to the observations of Andersson & associates (159), a report from another area presents evidence of an increasing incidence of scruvy in Nashville, Tennessee and its environs [Woodruff (161)]. It is based on the statistics of the Vanderbilt University Hospital and is attributed to nothing more than an inadequate intake of the vitamin.

A megaloblastic anemia which responded to ascorbic acid, and which occurred in association with clinical scurvy in a man with hepatic cirrhosis, is described [Brown (162)]. The explanation that the anemia was due in part to ascorbic acid deficiency and in part to an abnormal folic acid metabolism as a result of the liver disease appears logical in view of the known interrelationship between folic acid and ascorbic acid. The appearance of typical scorbutic changes in the long bones in an individual with widespread neuroblastoma following a course of therapy with the folic acid antagonist, Aminopterin, represents another case in which the folic-ascorbic acid interrelationship might apply [Dennis & Mercado (163)]. Treatment with massive doses of ascorbic acid following withdrawal of the antagonist brought about a favorable response in the bone changes. Oral therapy with extremely large doses of ascorbic acid resulted in a significant reversal of the insulin-glucose tolerance curve toward normal in a group of obese individuals [Arendt & Pattee (164)] with normal oral glucose tolerance curves. The effect of the

same therapy in patients with abnormal glucose tolerance was of questionable significance.

An increase in the urinary ascorbic acid excretion following the administration of certain B-vitamins, but a decrease following the administration of phosphorylated derivatives of these vitamins to patients with liver disease is interpreted as a deficiency of phosphorylation of B-vitamins in patients with hepatic disease [Barbieri (165)]. Alteration of ascorbic acid metabolism is attributed to the fact that it requires the energy liberated in the dephosphorylation of ATP for the oxidation of the vitamin.

Studies continue to be reported on the effect of controlled intakes of vitamin C in human adults on the ascorbic acid content of white cells and serum [Steele *et al.* (166)]. The average serum level was observed to be a relatively good indicator of the white cell level of the vitamin [Morse *et al.* (167)]. A saturation test based on the number of days required to produce excretion of 50 mg. of ascorbic acid during the fourth to seventh hr. after a test dose of 5 mg. of ascorbic acid/lb. body weight is claimed to give good correlation with the type of diet, and the initial blood ascorbic acid level [Rowlands *et al.* (168]. The average ascorbic acid content in mg. per cent of the milk and blood of 42 nursing mothers and the blood of their sucklings is given as 3.92, 1.23 and 1.16, respectively [Cutroneo (169)]. There was no correlation between the blood and milk levels. Intravenous injection of 300 mg. of ascorbic acid in nursing mothers resulted in increases in the vitamin C content of the milk, with peak values at the 12th hr. Adult and cord blood was hemolyzed by incubation *in vitro* in increased oxygen tensions [Räihä (170)] and increased levels of ascorbic acid in the blood were reported to have an inhibitory effect upon such hemolysis.

Morphological and metabolic changes in scurvy.—The ascorbic acid content of the liver and kidney was markedly reduced and the phosphatase activity decreased in the scorbutic guinea pig [Weatherhead (171)]. In addition, the periodic acid Schiff substance of the same tissues of the extremely scorbutic guinea pig was reduced as a result of the diminished glycogen content of the cells, and the mitochondria in most cells were enlarged, swollen, and many were clumped and coalesced. The fact that the collagen-poor repair tissue in vitamin C-deficient guinea pigs contains approximately five times as much aminomucopolysaccharide as the collagenous repair tissue in control guinea pigs is interpreted as an excess formation of mucopolysaccharides in scorbutic granulation tissue [Robertson *et al.* (172)]. The predominant mucopolysaccharide in this tissue was isolated and characterized as hyaluronate.

Administration of vitamin C prevents the decrease in RNA and DNA of the granulation tissue of guinea pigs kept on a scorbutogenic diet [Rudas (173)]. In animals treated with the vitamin prior to inflicting a wound, vitamin C was capable of increasing the nucleic acid content of the granulation tissue to normal levels, but even large doses failed to restore the DNA value to normal when administered after inflicting the wound. In studies

on the incorporation of P^{32} into the RNA- and DNA-phosphate of the spleens of normal and scorbutic guinea pigs, no differences were observed in the specific acitivities of the nucleic acids of the spleens of the two groups of animals under resting conditions [Klein & Swick (174)], but under hypoxic conditions, normal spleen showed a marked increase in the specific activity of both nucleic acids, while in the scorbutic spleen only the RNA exhibited an increase.

Thyroxine is known to have a retarding influence on wound healing, but this does not appear to be related to any thyroxine-ascorbic acid antagonism in the process of wound healing. [Moltke (175)]. Since thyroxine has no effect upon the tensile strength of the wound in scorbutic guinea pigs, ascorbic acid and thyroxine are believed to influence different phases of fibrillogenesis. Treatment of guinea pigs for 20 days with propylthiouracil resulted in an increase in the ascorbic acid content of the thyroid, while treatment with thyrotropin led to a decrease [Chiti *et al.* (176)].

Quantitative electrophoretic changes in the serum protein-fractions associated with a 20 per cent decrease in the albumin/globulin ratio occur in sorbutic guinea pigs [Chalopin (177)]. Each of the serum protein fractions exhibited a reduction in mobility, an effect opposite to that which has been observed in vitamin C-deficient monkeys [Greenberg *et al.* (178)]. Deficiency of the vitamin in the guinea pig prevents development of immunity to diphtheria during active immunization and lowers established immunity to this disease [Bersins (179)].

Capillary resistance which decreased gradually for 46 days in guinea pigs deprived of ascorbic acid was retarded by the administration of vitamin A and unaffected by vitamin D [Desmarais & McCraw (180)]. The anemia of scurvy in guinea pigs consisting of a reduction in erythrocytes, hemoglobin, thrombocytes, and an increase in leukocytes is the immediate consequence of vitamin C deprivation, and is postulated to be the result of a delay in erythrocyte maturation (181). Hyporeactivity of the contractile vessels accompanied by dilatation and slowing of the blood is described as the major derangement of the peripheral vascular system in guinea pig scurvy [McCraw (182)]. The decrease in thromboplastin is attributed to a thrombocytopenia and the hypothrombinemia to a secondary vitamin K deficiency.

Metabolism in micro-organisms and plants.—Oxidation of ascorbic acid by peroxide in the presence of Cu confers strong bactericidal, fungicidal and viricidal properties upon the resulting preparation [Ericsson & Lundbeck (183)]. The mechanism of the effect is not related to the formation of H_2O_2. When certain lactobacilli are grown on a chemically defined media, ascorbic acid in suitable concentrations or other reducing substances cause a disappearance of the requirement for cysteine [Koser & Thomas (184)]. Of five strains which behaved in this manner, one was *L. casei* and the other four were hetero-fermentative *L. fermenti.*

The synthesis of ascorbic acid by *Fusarium vasinfectum* is decreased in cultures deficient in Zn (185), thus suggesting that Zn plays a role in the

synthesis of vitamin C by this fungus. $(NH_4)_2SO_4$ nhibits the conversion of uniformly labeled C^{14}-glucose to ascorbic acid in pea seedlings [Sastry & Sarina (186)]. The ammonium compound inhibits only the utilization of glucose and not that of glucurono- γ-lactone as shown in the following reaction:

$$\begin{array}{c} NH_4^+ \\ \downarrow \\ \text{glucose} \rightarrow \text{glucuronic acid} \rightarrow \text{gulonic acid} \rightarrow \text{ascorbic acid} \end{array}$$

The 1956 review in the *Annual Review of Biochemistry* considered the scheme for the two alternate series of reactions by which ascorbic acid can be synthesized in the plant from glucose and galactose including a description of the enzyme from pea seedlings which catalyzes the reduction of D-galacturonic acid methyl ester. A more complete account of the latter is now available [Mapson & Isherwood (187)]. The observations of Loewus and associates (188), which follow offer no support for this scheme. Evidence from studies on the synthesis of ascorbic acid in ripening strawberries from stem-fed or injected D-glucose-1-C^{14}, D-glucose-2-C^{14}, galactose-1-C^{14}, sodium glucuronate-6-C^{14}, or glucuronolactone-6-C^{14} suggests strongly that the carbon chain of glucose is preserved intact in its transformation to ascorbic acid, but galactose enters the triose pool prior to conversion to the vitamin. The inversion of the glucose configuration does not occur during the process of transformation. An insignificant amount of the C^{14} of the other two compounds appeared in the ascorbic acid synthesized by the strawberry. The pathway for the synthesis of ascorbic acid from glucose in normal and chloretonized rats [Burns & Mosbach (189)] is in agreement with that described for the strawberry.

Chloretone-stimulation of vitamin C synthesis as judged by increased urinary excretion and tissue reserves of ascorbic acid is fully suppressed by the simultaneous administration of ATP, malic acid, or thyroxine [Ganguli *et al.* (190)]. These three compounds also restore the lowered tissue concentrations of phosphate esters which result from the administration of chloretone.

Relation to endocrines, stress or injury.—Previous studies have shown that when animals are subjected to various kinds of stress, such as cold, x-ray, and thermal shock, the excretion of ascorbic, dehydroascorbic, and diketogulonic acid is increased. When daily injections of ACTH, thyroid extract, and somatotrophic hormone were administered to rats in an attempt to determine the site of production of the two oxidation products of ascorbic acid, no greater excretion of the three compounds occurred with any of the three hormones than with physiological saline [Monier & Byer (191)]. Injected physiological saline itself induced stress and augmented the excretion of ascorbic acid and its oxidation products.

Within four hours after rats received 600 r of x-ray [Klein *et al.* (192)], thymus, lymph nodes, and spleen showed a drop in ascorbic acid concentration, and the ascorbic level continued to decrease throughout the period of

study. On the other hand, intestinal mucosa exhibited a substantial rise in ascorbic acid content during the first four hours, which was followed by a gradual decline. Other tissues studied revealed no significant changes in ascorbic content as a result of x-irradiation. The authors suggest that the increase in the concentration of ascorbic acid in the intestinal mucosa is the result of the inhibition of mitosis brought on by x-ray and that "ascorbic acid is requisite to and/or consumed by mitotic activity."

As a result of a study of the effect of stress induced by electrical stimulation of the cervical sympathetic trunk of the rat on the number of eosinophils, lymphocytes, and neutrophils, and on the total plasma ascorbic acid of blood, Allison (193) has concluded that the plasma ascorbic acid which rises temporarily following such stress appears to be the most reliable index of stress, although statistical analysis of his results indicates eosinopenia as the most sensitive indicator. Exposure of rats to high O_2 pressures induced stress and decreased adrenal ascorbic acid [Gerschman & Fenn (194)]. The excretion of 17-hydroxycorticosterone in the urine of guinea pigs fed a vitamin C-free diet for seven days was observed to be markedly increased [Oertel & Hein (195)]. With graded doses of ascorbic acid, the concentration of the vitamin was increased in the adrenals with each increase in the level of intake. Continued administration of cortisone caused a lowering of both the concentration and the total quantity of ascorbic acid in the adrenal glands. ACTH also lowered the concentration, but because the glands were greatly enlarged by the treatment the total quantity of vitamin C present in the glands was increased [Constable *et al.* (196)].

On the basis of *in vitro* studies of the hepatic metabolism of corticosteroid hormones, a mechanism for the participation of ascorbic acid in hepatic corticosteroid metabolism has been proposed [Bacchus & Debons (197)]. The ascorbic-dehydroascorbic acid couple is represented as a competitive hydrogen acceptor system which undergoes preferential reduction by liver in such a way as to inhibit the reduction of Δ^4, 3-ketones.

In the scorbutic gunea pig there is a quantitative and qualitative variation in corticosteroids which results from deviations in metabolism [Ratsimamanga *et al.* (198)]. Lack of ACTH at the pituitary level is not the explanation, nor is it the result of non-mobilization of corticosteroids from the adrenals. Despite the absence of any change in sensitivity to ACTH, the scorbutic adrenal, while able to synthesize corticosteroids, is not able to metabolize deoxycorticosteroid-type steroids well, and is presumed to convert these to polar toxic derivatives which the scorbutic animal is unable to use because of the lack of ascorbic acid. The vitamin plays a role in the hydroxylation, oxygenation, and oxidation of corticosteroids and helps to regulate the rates of the enzymic processes involved in these reactions. The authors postulate that the active form of the cortical hormones may be a complex of corticosteroid and water soluble vitamins, including ascorbic acid and pituitary substances.

Pre-treatment of intact rats with ascorbic acid at levels of 50 to 250

mg. / 100 gm. body weight did not modify the response of the blood glucose to insulin, and failed to affect other parameters of carbohydrate metabolism and the cholesterol concentration of the adrenals [Dury & Dury (199)]. Contrary to an earlier observation (200), no loss in activity of ACTH occurred as a result of incubation with ascorbic acid [Dedman *et al.* (201)]. Glycine potentiates the salicylate-induced depletion of ascorbic acid in Wistar rats, but not in Tennessee Medical Unit rats [Eades & King, Jr. (202)]. Experimental hemorrhage produces a lowering of the ascorbic acid content of the adrenal cortex, which to some extent is proportional to the severity of the hemorrhage [Blamoutier (203)].

Massive doses of ascorbic acid in man did not affect significantly the metabolism of a small oral dose of hydrocortisone as measured by alterations in plasma hydrocortisone and urinary 17-hydroxycorticoid and 17-ketosteroid levels of excretion [Haynes & Sheid (204)], results which are opposite to those observed in the rat by Bacchus *et al.* (205). Administration of ascorbic acid is claimed to lead to increased synthesis and a sparing action (to the extent of 30 to 40 per cent) on corticosteroids [Seliger (206)].

Chondroitin sulfate synthesis as measured by the incorporation of S^{35} labeled sulfate into costal cartilage was strikingly inhibited by cortisone in guinea pigs on a relatively high intake (50 mg./100 gm. body weight/day) of ascorbic acid [Cheng & Shank (207)]. Also guinea pigs receiving the minimum daily requirement of the vitamin synthesized twice as much chondrotin sulfate as animals which received no supplement. The results of Upton *et al.* (208) on the utilization of S^{35} labeled sulfate by healing wounds in scorbutic and non-scorbutic guinea pigs exhibit a similar relationship.

In intermediary metabolism.—An enzyme system of dog liver catalyzes the oxidation of *p*-hydroxyphenylpyruvic acid to homogentisic acid [La Du & Zannoni (209)]. Resolution of the enzyme system into two protein fractions, both of which are required for the oxidation, has been accomplished. One fraction has been identified as catalase. Following purification of the enzyme system, the oxidation of *p*-hydroxyphenylpyruvic acid was no longer stimulated by vitamin C, but 2,6-dichlorophenolindophenol still remained effective. On the basis of the last observation, it has been suggested that ascorbic acid acts indirectly through some substance which is inactivated or lost during purification. Injection of NaN_3 decreases the activity of a similar enzyme in rabbit liver [Ikeda *et al.* (210)]. which is reactivated by vitamin B_{12} and to some extent by ascorbic acid. The excretion of homogentisic acid by scorbutic guinea pigs fed tyrosine or phenylalanine was prevented by the administration of choline or glycine [Malakar & Banerjee (211)]. Scorbutic guinea pigs fed L-tyrosine and injected intraperitoneally with sodium butyrate developed cataracts and excreted large amounts of benzoquinoneacetic acid in the urine [Uyama *et al.* (212)], but the excretions of homogentisic acid and *p*-hydroxyphenylpyruvate were normal. The administration of benzoquinoneacetic acid also induced cataracts, but feeding tyrosine alone did not lead to cataracts.

Similar studies on a tyrosine-oxidizing system have now been extended to the liver of man [Kretchmer *et al.* (213)]. The activity of the system is 10 to 30 times greater in the adult liver than it is in that of the full-term infant and 3 to 5 times greater in the latter than it is in the liver of the premature infant. *In vitro* addition of ascorbic acid failed to stimulate activity in the premature infant liver but did so in the adult liver. Evidence suggests lack of the apoenzyme in the premature infant during the first few days of life with rapid formation occurring subsequently.

An enzyme which converts kynurenine to 3-hydroxykynurenine has been obtained in partially purified form from cat liver by Ito (214). The enzyme was inactivated by dialysis and could be reactivated by the addition of ascorbic acid and vitamin B_{12}. The biosynthesis of glycine or the synthesis of hippuric acid and formate from serine in the guinea pig were unaffected by the deficiency of vitamin C [Arnstein & Stankovic (215)].

Ascorbic acid completely inhibits the spontaneous oxidation of adrenaline in neutral or slightly alkaline solution, but only partially inhibits its decomposition by guinea pig liver preparations [Malafaya-Baptista *et al.* (216)]. Results which contradict the Lazarow's sufhydrul theory of diabetes (217) have been obtained on rats rendered permanently diabetic by the administration of dehydroascorbic acid [Bhattacharya *et al.* (218)]. The diabetes did not alter the concentrations of reduced glutathione in blood, liver and pancreas. Contrary to the results of Princotto (219) the authors were unable to induce diabetes in rabbits by intravenous injection of dehydroascorbic acid.

Evidence from *in vitro* studies of homogenates of the adrenals from scorbutic and non-scorbutic guinea pigs suggests that ascorbic acid is involved in the biosynthesis of corticosteroids from cholestenones [Rahandraha *et al.* (220)]. Homogenates of normal guinea pig adrenals rich in ascorbic acid lose their cortin-like properties much more slowly than that of scorbutic adrenals. When the incubation is carried out in the presence of cholestenones, the formation of polar steroids of the cortisone- and corticosterone-type are produced with homogenates of normal adrenals, but this does not occur with homogenates of scorbutic adrenals. The incorporation of acetate-1-C^{14} into cholesterol and fatty acids during a three hour incubation period by isolated adrenal, aorta, and liver of guinea pig was unaffected by a deficiency of ascorbic acid [Bolker *et al.* (221)]. The observation on liver tissue is at variance with that reported by Becker *et al.* (222) who found the specific activities for cholesterol and fatty acid in the scorbutic guinea pig liver after a one hour incubation period to be double those of the non-scorbutic liver, but just the opposite after a two and three hour incubation period.

Catabolism.—Differences in the metabolism of ascorbic acid in the guinea pig and in man are revealed by studies with C^{14}-labeled ascorbic acid [Burns *et al.* (223)]. In the guinea pig only a small fraction of the vitamin is excreted in the urine as the unaltered vitamin, diketogulonic acid, and oxalic acid. The major portion undergoes oxidation of its entire carbon chain to CO_2. The half life in the guinea pig is about four days. On the other hand, in man,

little C^{14} appears in the respiratory CO_2, and nearly all of the C^{14} is eliminated in the urine as ascorbic, dehydroascorbic, diketogulonic, and oxalic acids. The fact that the half life in man (approximately 18 days) is considerably longer than that of the guinea pig accounts for the greater length of time required for the development of scurvy in man.

Functions.—A recent investigation offers support for the long-standing theory that ascorbic acid plays a role as a respiratory catalyst. Glutathione and ascorbic acid, act as respiratory carriers in the respiration of pea seedlings [Mapson & Moustafa (224)]. Extracts obtained from germinating pea seeds contain an enzyme system which transfers H to molecular O_2 from substrates of TPN-linked dehydrogenases (isocitrate and malate). The enzymes participating in the respiratory pathway include dehydrogenase enzymes, glutathione reductase, dehydroascorbic acid reductase and ascorbic acid oxidase. More evidence for a catalytic role of ascorbic acid in electron-transport is presented [Kersten *et al.* (225)].

Since a bound form of vitamin C may be intimately related to its function, the question of its existence in tissues becomes of real importance. The presence of bound ascorbic acid in the liver of the guinea pig first reported by Sealock and coworkers (226, 227) has now been confirmed [Dayton *et al.* (228)], by the administration of L- and D-ascorbic-1-C^{14} to guinea pigs. Approximately 19 per cent of the liver L-ascorbic acid is bound in normal and 28 per cent in scorbutic guinea pigs. The D-enantiomorph was found to be bound in the liver of the ascorbic acid deficient guinea pig to about the same extent as L-ascorbic acid. It will be recalled that Sealock and associates (226) had attributed considerable importance to the bound form of ascorbic acid in connection with the metabolism of tyrosine. The presence in animal cells of a bound complex of ascorbic acid, Fe, and nucleic acid in which the Fe is located between the phosphate and ascorbic acid is described [Gol'dshtein (229)]. It is claimed to convert RNA of the cytoplasm to DNA of the nuclei.

Interrelationship with other vitamins.—The cocarboxylase content of heart, liver, striated muscle, brain, and uterus was decreased in scorbutic guinea pigs in proportion to the degree of ascorbic acid deficiency [Barbieri (230)]. Although the quantity of acetylated PAB excreted in the urine of scorbutic guinea pigs was less than that of pair-fed controls, the CoA activity of liver, kidney, and brain did not differ in the two groups of animals [Lahiri *et al.* (231)]. Administration of 40 mg./day of Aminopterin decreased the urinary excretion of ascorbic acid in normal rats but had no influence on the chloretone-treated rat [Lahiri *et al.* (232)]. Conversion of folic acid to citrovorum factor is increased in the latter. The explanation may be related to the enhanced biosynthesis of ascorbic acid and glutathione in the chloretonized animal. The storage in the liver has been used as a measure of influence of ascorbic acid on the conversion of carotene to vitamin A in the rat [Mayfield & Roehm (233)].

Oxidizing enzymes & oxidation.—A non-specific enzyme which catalyzes the oxidation of ascorbic acid by O_2 and also the oxidation of hydroquinone,

p-phenylenediamine, guiacol and pyrocatechol is present in *Agaricus campestris* [Legrand (234)]. The purified enzyme is of the laccase type which oxidizes vitamin C directly and shows no requirement for an intermediary carrier. Its activity is not related to Fe or Cu. Ascorbic acid oxidase has been prepared from *Citrullus vulgaris* [de Farias & Neto (235)]. Nasunin, the anthocyanin pigment of the pericarp, and a globulin of the sarcocarp of egg apples are inhibitors of cucumber ascorbic acid oxidase. The protein acts by combining with the prosthetic group [Inagaki *et al.* (236)].

Autooxidation of L-ascorbic acid at pH 7.1 is accelerated by imidazole carboxylic acid and histamine, but is inhibited by methylimidazole. The addition of Fe suppresses slightly the autooxidation and counteracts the inhibition of methylimidazole [Imanaga (237)].

Photochemical oxidation of ascorbic acid by ultraviolet was investigated in solutions which contained oxalic acid [Baker *et al.* (238)]. The oxidation was catalyzed by Fe, and when adequate oxygen was present, the amount of the vitamin oxidized was proportional to the time of irradiation and independent of the initial concentration in the range tested. No oxidation of ascorbic acid occurred in the dark when O_2 was excluded or in the absence of Fe.

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WATER-SOLUBLE VITAMINS, PART III[1,2]

Pantothenic Acid, Thiamine, Lipoic Acid (Thioctic Acid), Riboflavin, and Inositol

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Pantothenic Acid

The pantothenate requirement necessary for normal reproduction of female swine is 12.5 mg. per kg. (1), which is similar to the requirement for maintaining optimum growth and feed efficiency in baby pigs (2). A high incidence of duodenal ulcers occurs in rats placed on a pantothenate-deficient diet after they are 30 to 35 weeks old, but not in those placed on the deficient diet at weaning (3).

When mouse stomachs are incubated *in vitro* with N-ethylmaleimide (0.25 to 1.0 m*M*) for 10 min., the CoA content of the stomachs and also gastric secretion are significantly reduced (4). The addition of 30 to 150 units of CoA per ml. restored gastric secretion to normal. Benemide, *p*-(dipropylsulfamyl)benzoic acid, which had been shown by Schachter & Taggart (5) to inhibit renal transport mechanisms presumably by blocking the formation of benzoyl CoA, likewise caused inhibition of gastric secretion. Because of other experiments suggesting that N-ethylmaleimide may act at least in part by reducing the availability of high energy phosphate bonds, Davenport *et al.* (4) thought that the role of CoA in gastric secretion might involve its function in the supply of energy to the tissue. The CoA antagonist D-(pantoyltauryl)*p*-aniside had no effect on gastric secretion.

Braekkan (6) studied the changes in pantothenic acid content in ovaries of the cod (*Gadus morrhua*) during the reproductive cycle. In ovaries of juvenile and mature fish in the first stages of regeneration, pantothenate values up to 375 μg. per gm. wet weight or 2320 μg. per gm. dry weight were obtained. The fish ovary thus supersedes royal jelly as the natural material

[1] This review covers the papers that appeared through December 1, 1956 in the journals available to the author. Because of space limitation, the review could not be all-inclusive, but rather certain areas of investigation were emphasized; thus some areas have been neglected.

[2] The following abbreviations are used: ACTH for adrenocorticotropic hormone; ATP, ADP, and AMP for adenosine tri-, di-, and monophosphate; CoA for coenzyme A; CoASH for reduced coenzyme A; DPN and DPNH for the oxidized and reduced forms of diphosphopyridine nucleotide; FAD for flavinadenine dinucleotide; FMN for riboflavin-5′-phosphate; PABA for *p*-aminobenzoic acid; OYE for old yellow enzyme; TPN and TPNH for the oxidized and reduced forms of triphosphopyridine nucleotide, and TPP for thiamine pyrophosphate or cocarboxylase.

[3] Operated by Union Carbide Nuclear Company for the Atomic Energy Commission.

containing the highest amount of pantothenic acid (7). During the regenerative or reproductive cycle the panthothenic acid content of the ovaries steadily decreases, reaching a minimum value of 10 to 30 μg. per gm. wet weight shortly before spawning. Riboflavin and vitamin B_{12} did not show similar changes. Since only the free pantothenate content of the ovaries was measured, one wonders if the decrease in free pantothenate during the reproductive cycle might be caused by its conversion to CoA.

Boccacci & Bettini (8), using the American cockroach (*Periplaneta americana* Linnaeus) and the housefly (*Musca domestica* Linnaeus), found in the former 11.4 units of CoA per gm. of whole coxae (fresh weight) and 6.1 and 28.0 units for white and red coxal muscle, respectively. The CoA content of whole flies increased from 2.3 units per gm. fresh material on the first day of age to 7.7 units on the ninth day, then declined to 6.5 units per gm. by the twelfth day of age. Since a similar curve of increase has been seen for the muscle sarcosomes, the authors conclude that a high CoA content indicates a more highly developed oxidative metabolism.

The pantothenic acid of human colostrum and breast milk was determined for 38 women by Pellegrini & Chiari (9). The subjects varied in their time of onset of lactation from 1 day to 11 months. In general, the pantothenate content of the milk was low on the first day (average 1.55 μg. per ml.), reached a maximum of 3.0 μg. per ml. by the tenth day, and remained at this elevated level through 11 months. Digestion of the milk with papain and takadiastase led to an increase of 23 per cent in the amount of free pantothenate, indicating that a portion of the pantothenate in milk is bound in a form not available to the assay organism [see also Schmidt (10)]. It is interesting to compare these results with those of Ringler *et al.* (11), who found a seven-fold increase of CoA in the lactating mammary gland of the guinea pig 6 days postpartum. CoA gradually declined to normal values after about 46 days. Read & Moore (12), pursuing the latter observation, reported that the injection of prolactin into lactating guinea pig mammary glands *in situ* significantly increased CoA concentration within 1 hr. after injection. Since CoA is degraded at an extremely rapid rate in homogenates of lactating mammary tissue, it was suggested (12) that prolactin inhibits this degradation of CoA.

Boxer *et al.* (13), in a comprehensive study of the effect of vitamin B_{12} deficiency on the CoA content of tissues, found a two- to three-fold increase in the CoA content of the liver and kidney, but not of the brain, in rats made deficient in the vitamin. The increase in CoA content is readily reversible since restoration of adequate amounts of vitamin B_{12} by stomach tube resulted in a return to normal CoA levels in 2 to 4 days. The elevated hepatic CoA of B_{12}-deficient rats is not caused by a decreased rate of destruction of CoA, nor to a shift in the ratio of oxidized to reduced CoA. Analysis of a number of CoA-catalyzed enzymatic reactions failed to reveal any significant differences between the normal and B_{12}-deficient tissues. Since Oginsky *et al.* (14) had shown that an *Escherichia coli* mutant culturable only with

vitamin B_{12} or methionine had markedly reduced rates of oxidation of acetate and glutamate when grown with methionine, Boxer *et al.* (13) examined the CoA content of such cultures. No significant differences in CoA content between the vitamin B_{12}- and methionine-grown cultures was found. In contrast to these studies, Jännes (15) isolated several strains of *E. coli* from human feces and found that vitamin B_{12} at a concentration of 0.3 μg. per ml. of medium inhibited markedly the synthesis of pantothenic acid. Addition of vitamin B_{12} (1.0 μg. per ml. of medium) had no influence on the CoA content of the cells. Since the excretion of free pantothenate into the culture medium is severely inhibited by this concentration of vitamin B_{12} one can infer that these organisms must synthesize much more pantothenate than they need for the synthesis of their CoA requirements. The two- to threefold rise in liver CoA with vitamin B_{12}-deficient rats was confirmed by Wong & Schweigert (16), who suggested that, since the vitamin B_{12}-deficient rat cannot utilize carbohydrate efficiently [Ling & Chow (17)] the rise in liver CoA may be a physiological adaptive mechanism that increases energy production by providing more C_2 units from fatty acid oxidation. In any case, this observation is of particular interest since it suggests some hitherto unsuspected role for CoA.

Garattini *et al.* (18) reported that 6-mercaptopurine and thioguanine inhibit the *in vitro* acetylation of sulfanilamide and also the growth of Walker carcinoma. Since the latter inhibition can be prevented by either ATP or pantothenate the authors suggest that the mercaptopurine and thioguanine effects are attributable to inhibition of CoA. Scorbutic guinea pigs had a decreased ability to acetylate an injected dose of PABA (19). The tissues, however, had a normal CoA content, indicating that the decreased ability to acetylate is not caused by a decrease in CoA. Navazio & Siliprandi (20) showed that the acetylating ability of human blood is essentially normal in uncomplicated diabetes, but virtually absent in diabetic coma. Unfortunately, no attempt to correlate this finding with other CoA activities was made; thus it is difficult to assess the nature of the inhibitory effect on the acetylation process.

The history of the development of information about the structure of CoA has been reviewed by Baddiley (21), who documents the chemical evidence supporting the present formulation of the structure. Wieland *et al.* (22) prepared C^{14}-pantothenate, labeled in the 3 position of β-alanine. The labeled pantothenate was fed to *Streptobacterium plantarum* and to *Saccharomyces carlsbergensis*. In the lactic acid organism pantothenate, 4′-phosphopantetheine, pantothenylcysteine, dephosphoCoA, and CoA were identified, confirming the biosynthetic pathway proposed by Novelli (23). In the yeast, however, they found pantothenate, 4′-phosphopantothenate, and CoA, suggesting the presence of an alternative route in the biosynthesis of CoA [see Brown & Snell (24)]. Cummins *et al.* (25), reported on the probable occurrence of pantothenylcysteine in the beef liver; this suggests that the former pathway for CoA biosynthesis (23) may be operating in this tis-

sue. Trufanov & Popova (26) demonstrated the net synthesis of CoA from pantothenate, ATP, and cysteine in brain homogenates, but not in homogenates of liver, kidney, or muscle, presumably because of rapid enzymatic destruction of CoA by the latter tissues. Thiamine and pyruvate were reported to increase CoA synthesis in the brain homogenates. CoA biosynthesis in homogenates of benign human cerebral tumors was more intense than in homogenates of malignant tumors. Total-body irradiation of guinea pigs with 500 r led to a decrease in the ability of brain homogenates to synthesize CoA. That this is not caused by destruction of CoA by radiation is evident from the report of Gardella & Lichtler (27), who found that 10,000 r delivered *in vitro* to Yoshida ascites tumor cells did not alter significantly the CoA content, nor did 1000 to 1600 r of total-body radiation alter the CoA levels of the liver or thymus of the Wistar rat.

Leuschner (28) examined the CoA-containing compounds of guinea pig liver by acetone fractionation and paper chromatography. Seven CoA-containing compounds, CoASH, acetylCoA, and five combinations of CoA with sulfur-containing substances whose —S—S— bond could be broken with KCN were found. Brenner-Holzak *et al.* (29) have carefully worked out chromatographic procedures that gave good separation of CoA, dephosphoCoA, and also —S—S— forms. Small quantities of CoA were separated by paper chromatography (solvent system 1 per cent Na acetate:propanol 1:1). For large-scale separation they used Dowex-1×4 and gradient elution [Hurlbert *et al.* (30)] with a mixture of acetic acid and ammonium acetate. Using these methods, they found that Pabst CoA contained, mainly, reduced CoA, but they also found oxidized CoA, dephosphoCoA, ADP, and ATP. With the purified forms of CoA and dephosphoCoA, Brenner-Holzak & Leuthardt (31) were able to compare the activities of these materials in a variety of enzyme systems. For the oxidation of α-ketoglutarate, dephosphoCoA can replace CoA only when used in high concentration (0.1 to 0.2 μmole per ml.), whereas 4′-phosphopantetheine is totally inactive. AcetylCoA and acetyldephosphoCoA are equivalent acetyl donors for the acetylation of *p*-aminoazobenzene with a purified pigeon liver enzyme. Acetyl-4′-phosphopantetheine was totally inactive in the latter system.

FluoroacetylCoA was synthesized by Brady (32), and Marcus & Elliott (33) and its reactivity compared with acetylCoA in a number of enzyme systems. Brady (32) reported that fluoroacetylCoA is metabolically active with phosphotransacetylase, thioltransacetylase, and arylamine acetylase. It is active in the acetoacetate synthesizing system and condenses with oxaloacetate to form fluorocitrate. It is inactive, however, with the acetate-activating enzyme from pigeon liver, which explains the report of Peters (34) of the inability of pigeon liver preparations to form fluorocitrate from fluoroacetate. An enzyme that can activate fluoroacetate is present in rabbit kidney.

Tarbell & Cameron (35) synthesized β-(N-methylacetamide)-ethylthioacetate, N,S-diacetylaletheine, and γ-acetaminopropylthioacetate as thio-

esters related to CoA. They found that the rates of alkaline hydrolysis and aminolysis of these compounds do not differ significantly from those with ethyl thioacetate. Sakuragi & Kummerow (36) synthesized ethyldipalmitoxypantothenate and ethylmonopalmitoxypantothenate and found such esters of pantothenic acid readily soluble in fat and apparently easily hydrolyzed since they showed full activity with rats as a source of pantothenate (37). Stewart *et al.* (38) synthesized eight new analogues of β-aletheine and pantetheine and tested them microbiologically for growth promotion or inhibition with several microorganisms. Examination of the activity of these compounds suggested that, in addition to the thiol group of CoA, the biological activity of pantetheine and CoA may also be dependent on two amide bonds in the molecular structure, probably as points of attachment to the protein. N-pantothenylethanolamine (oxypantetheine) proved to be a competitive inhibitor with *Lactobacillus helveticus*, whereas high concentrations of this compound were able to support the growth of *L. fermenti*. Boxer *et al.* (39) studied the effect of pantoylaminoethanethiol (PAET) as an antagonist of pantetheine in the synthesis of CoA. PAET completely inhibited the synthesis of CoA from pantethenine in pigeon liver extracts at a molar ratio of 1:250. Fifty per cent inhibition occurred at a molar rate of 1:4 to 1:30. PAET also inhibited the metabolic function of CoA in the phosphotransacetylase system, but required a higher concentration, i.e., molar ratio of 1:5000. PAET (10 mg. per day) fed to rats on a pantothenate-deficient diet led to deaths beginning on the eighteenth day, and by the twenty-fifth day all animals were dead. Death apparently occurs as soon as the liver CoA content is decreased to one half of its normal value. The CoA content of the brain, however, remained normal even in the most severely deficient animals. This appears to be similar to the failure of brain CoA to respond to B_{12} deficiency (13).

Shils *et al.* (40) determined the CoA content of 755 tumor and nine normal tissues or organs of the mouse. They found the following percentages of CoA relative to the liver, 100; kidney, 84.9; heart, 59.6; stomach, 25.7; brain, 22.8; testis, 22.6; lung, 12.4; muscle, 6.8; and tumor, 11.5. Thus, except for muscle, tumor tissue had the lowest content of CoA. This observation confirms and extends the report of Higgins *et al.* (41) that rat liver tumor contains less CoA than normal liver.

Bean and his colleagues (42) have repeated their previous investigations (43) of pantothenic acid metabolism in human subjects but with considerable improvement in the design of experiments. In spite of the fact that the subjects experienced symptoms that have also been observed in animals on a pantothenate-deficient diet, the failure of many of the abnormalities to disappear when pantothenate alone was restored to the diet (but instead, required a complete vitamin supplemental diet) raises the question as to whether the abnormal symptoms are caused solely by pantothenate deficiency or whether ω-methylpantothenate is a more powerful toxic agent working as a general protoplasmic poison. In this connection it is interest-

ing that Pudelkewicz & Roderuck (44) reported that feeding ω-methylpantothenate to guinea pigs led to a chronic rather than acute state of pantothenate deficiency.

It seems well established that pantothenate deficiency in the rat leads to a deficiency in adrenal cortical hormones (45, 46). The situation in the dog, however, is much less clear. Arnrich *et al.* (47, 48) have attempted to study the influence of pantothenate and riboflavin deficiencies on a number of adrenal functions in the dog. Dogs were made pantothenate-deficient and then subjected to stress by being forced to swim for 25 min. at 25°C. Blood glucose levels were followed throughout the study and the effects of fasting, epinephrine, and adrenal cortical extracts also recorded. There was a rise in blood sugar of 34 mg. per cent in normal dogs and 36 mg. per cent in pantothenate-deficient animals immediately after stress. Two hours after stress, the blood sugar level of normal aniamls had returned to the initial levels, but the pantothenate-deficient animals were now 14 mg. per cent below the prestress levels. Adrenocortical extracts diminished the postress rise in both normal and pantothenate-deficient animals and caused recovery from pantothenic acid-deficient hypoglycemic collapse. The authors conclude that there may be a deficiency of adrenal cortical hormones in the pantothenate-deficient dog.

Longwell & Hansbury (49) observed a 40 per cent decrease in corticosteroid output from the adrenal vein in rats made deficient by a combination of pantothenate-deficient diet and feeding ω-methylpantothenate. Barboriak (50) observed that the pantothenate-deficient, hypophysectomized rat responded to growth hormone only half as well as the hypophysectomized control. This stimulation of the pantothenate-deficient animal with growth hormone led to a high rate of mortality with congested and hemorrhagic adrenals. It was concluded that some mechanism other than a pituitary-mediated stress situation is responsible for the adrenal damage in pantothenic acid-deficient rats.

The effects of pantothenate deficiency on the diabetic status of the partially depancreatized diabetic rat have been reported by Hazelwood *et al.* (51). Insulin sensitivity in the intact or the partially pancreatized rats varied with the duration of pantothenic acid deficiency. In intact rats, eight weeks of deficiency resulted in some resistance to insulin, which increased after 14 weeks of the deficient diet. This was interpreted as indicating adrenal cortical hyperfunction early in the deficiency, and adrenal cortical hypofunction as the deficiency progressed. This confirms the work of Winters *et al.* (52).

Mookerjea & Sadhu (53) observed that diabetic rats fed an excess of pantothenate excreted less sugar and ketone bodies, and lost no weight. Cholesterol increased markedly in the liver, adrenals, and blood of normal and diabetic rats fed an excess of pantothenate. The authors suggest that the excess pantothenate may guide the metabolism of unoxidized acetate and overproduced acetoacetate toward increased synthesis of cholesterol in the diabetic animals. Rossi *et al.* (54) found no varition in production rate of

acetylCoA in liver tissues of rats made diabetic with alloxan, and suggest that animals poisoned with alloxan exhibit increased fatty acid oxidation because there is an availability of acylCoA spared in other metabolic processes whose rate is diminished. No suggestion was made, however, as to which process these might be. Brady *et al.* (55) obtained some interesting results on the effect of citrate and CoA on fatty acid metabolism that may now permit a more detailed interpretation of the increased oxidation of fatty acids, decreased biosynthesis of fatty acid (56, 57) and increased synthesis of cholesterol in diabetic animals (58). It has been known for some time that the presence of citrate caused a great stimulation of the incorporation of C^{14}-labeled acetate into long-chain fatty acids by a water-soluble enzyme system from pigeon liver (59). Brady *et al.* (55) have now established that the stimulatory effect of citrate is caused by the formation of TPNH in the oxidation of isocitric acid. The stimulatory effect of citrate appears to be explained by Langdon's finding (60) that the reduction of crotonylCoA to butyrylCoA by rat liver has an absolute requirement for TPNH. High concentration of CoASH seems to stimulate oxidation of fatty acid on the one hand and inhibit its synthesis on the other. They report a personal communication from Gordon Tompkins that the CoASH content of the liver of diabetic and starved animals is considerably higher than that in normal animals. Since the diabetic animal must burn fatty acid for energy, an increased amount of CoASH is necessary. The oxidation terminates with acetylCoA. Regeneration of CoASH presents a problem, since normal regeneration by condensation with oxaloacetic acid to form citrate is slowed owing to the decreased supply of oxaloacetic acid from the inhibited glucose metabolism. The latter phenomenon results indirectly in a decreased supply of TPNH, which inhibits the synthesis of fatty acid. The main task for the diabetic animal is thus to regenerate CoASH from acetylCoA to permit the continued oxidation of fatty acid. This can be accomplished through deacylation of acetoacetylCoA, which thereby liberates free acetoacetic acid (and the ketone bodies derived therefrom) and CoASH. Perhaps a more beneficial method of regenerating CoASH is to convert acetylCoA to cholesterol, and the report of Mookerjea & Sadhu (53) of increased synthesis of cholesterol by the diabetic animal fed an excess of pantothenate is consistent with these ideas. Bartlett and co-workers (61) reported that the CoA content of the liver, but not the kidney, of immature hypophysectomized rats was significantly lower than in the control animals. This confirms a previous report by Ringler & Leonard (62). Injection of growth hormone failed to simulate an increase in liver CoA of hypophysectomized animals. Chernick & Moe (63), however, were unable to confirm the effect of hypophysectomy on the CoA content of rat liver. In the latter study, the liver CoA content of hypophysectomized rats did not differ from that of unoperated controls of the same weight. One significant difference between the two investigations that might explain the divergent results is that, in the two former investigations, liver CoA was measured 10 days after operation; Chernick & Moe

waited 166 days after the operation to make the CoA measurements.

Yang *et al.* (64) studied the effect of CoA precursors on liver CoA levels in rats deficient in pantothenic acid or organic sulfur. In the synthesis of CoA, the latter have a more sluggish response to cystine than do the former to added pantothenate. The pantothenate-deficient animals responded to pantothenate, pantetheine, pantethine, pantothenylcysteine, and pantothenylcystine. These compounds were not effective in the organic sulfur-deficient rats.

Severi & Fonnesu (65) showed that when adult female rats are made to form fatty livers by CCl_4 intoxication, the CoA content of the liver is decreased by 33 per cent. The authors suggest that the low levels of CoA and ATP necessary for fatty acid activation would lead to impaired utilization of lipides in the liver, and consequently to a pathological accumulation of fat.

Thiamine

Beal (66) found that the thiamine intake of children rises from 0.30 mg. per 1000 kcal. at age one month to 0.70 mg. per 1000 kcal. by one year, decreases in the next two years to a level of 0.54 mg. per 1000 kcal, and then slowly rises again to 0.81 mg. per 1000 kcal. at 5.0 years of age. The decrease between one and three years of age is a reflection of the gradual increase in total calories during the period when thiamine intake remains unchanged.

Warnick *et al.* (67) observed that human subjects excreted less thiamine when potatoes furnished about one third of the dietary thiamine than when it was furnished by bacon, rice, lamb, lamb and potatoes together, or pure thiamine. They found that, when potatoes or brown rice replaced a portion of the grain in the diet of weanling pigs, the ham, loin, and shoulder cuts had a higher content of thiamine than those fed the rice diet. Thiamine content of heart, liver, and kidney did not vary with diet. These observations suggest that unknown factors associated with source of dietary thiamine may influence the utilization and deposition of thiamine in the tissues.

Luckey *et al.* (68, 69) have reviewed the work on vitamin metabolism in germ-free animals. The nutritional requirements of germ-free animals are qualitatively the same as those for conventional animals. Studies on the metabolism of germ-free rats with respect to food and water intake, growth rate, and reproduction, led to the statement: "It is possible that the net result of (microbial) intestinal synthesis may be disregarded as a nutritional factor." A complete vitamin balance study was run in germ-free rats fed a synthetic diet. In general, three different states of vitamin metabolism were seen. In one, less vitamin was recovered in the excreta, waste food, lost hair, and carcass than was put in with food. These groups of vitamins, containing niacin, riboflavin, and biotin, were evidently destroyed or changed during metabolism. With a second group of vitamins, including pantothenate and vitamin B_{12}, the balance between intake and output was the same. In the third group, exemplified by vitamin B_6, more of the vitamin was recovered than was fed, indicating a net synthesis.

Dellweg & Bernhauer (70) described an improved microbiological assay for thiamine that makes use of *L. fermentii* ATCC 9338. A linear response is given over the range 1.6 to 13.33 mμg. of thiamine per ml. Ohnesoige & Rogers (71) utilized the fluorescence of thiochrome at 450 mμ and of riboflavin at 530 mμ to devise a method of assaying these two vitamins in mixtures. By suitably controlling the pH, they used the method to measure mixtures of both vitamins ranging from 0.5 μg. per ml. with a precision of 5 per cent. Kay & Murfitt (72) improved the method of assaying TPP in blood by using a Norit-treated apoenzyme to reduce the blank, by the elimination of iodoacetate because of its possible inhibitory effect on the enzyme carboxylase, and by the addition of cyanide to the assay system. With these modifications, CO_2 production was linear with 0.05 to 2.0 μg. of TPP. Chen & Peppler (73) compared the relative baking activity of active dry yeast with the ability of the yeast to effect anaerobic fermation and pyruvate decarboxylation as a function of time of exposure to elevated temperatures. The decline in baking activity after exposure to a temperature of 48°C. was correlated with a decline in pyruvate decarboxylation activity that could be shown to be caused by destruction of cocarboxylase. Addition of TPP, Mg^{++}, and ATP restored pyruvate decarboxylation activity to normal. Suomalainen *et al.* (74) observed that the addition of thiamine to commercial bakers yeast accelerated carbon dioxide evolution from glucose, and also from fructose and mannose as well. But added thiamine did not abolish the usual induction period characteristic of maltose fermentation. Trevelyan & Harrison (75) continued their studies on the effect of thiamine on yeast metabolism and observed that the accelerating effect of thiamine on fermentation rate was dependent on the presence of a source of assimilable nitrogen. The increased fermentation rate, so induced, could be maintained only if phosphate was also present in the medium. When yeast is fermenting glucose in a thiamine-free medium, the intercellular pyruvate concentration is about 15.1 μM per gm. of yeast. The addition of thiamine reduced this level to 2.5 μM per gm. within 10 min. which is maintained rather constantly thereafter. Fermentation of glucose in complete medium resulted in a pyruvate concentration of about 2.5 μM per gm. Because the increase in fermentation rate induced by thiamine is dependent on a source of nitrogen, the authors suggest that the effect of thiamine is on some synthetic reaction. The reduction in rate of protein synthesis when thiamine is omitted may be caused by Interference with the incorporation of amino acids into protein by the high intercellular concentration of pyruvate or alanine.

Navazio & Siliprandi (76) report that liver homogenates from alloxan-diabetic rats metabolize less pyruvate and form less citrate when incubated with ATP and oxaloacetate. The addition of TPP increases both pyruvate disappearance and citrate formation in normal liver homogenates as well as in the diabetic homogenate; but, significantly, the values with the diabetic homogenates are now equivalent to those observed in the normals treated with TPP. It has also been reported (77) that the diminished rate of pyru-

vate oxidation in liver homogenates of rats treated with pyrithiamine can be increased to normal by the addition of TPP and TPP disulfide. By measuring the TPP content of the livers of normal and diabetic rats, Rossi *et al.* (78) confirm the observation that alloxan diabetes depresses liver TPP to about 50 per cent of the control animals. If, however, fructose is administered to alloxan diabetic rats, the fall in liver cocarboxylase is not seen. The authors suggest that ATP made available by the normal metabolism of fructose in the diabetic animal is capable of maintaining normal levels of liver TPP in such animals. In continuing studies on the biochemical action of polyphosphoric ester amides of thiamine (EAPP) Roux *et al.* (79, 80) reported that sodium di- and triphosphate and EAPP inhibit hexokinase and TPP, but not tri- and tetrametaphosphates, suggesting that only linear phosphate esters can show this effect. It is also reported that EAPP inhibits glycolysis in defibrinated blood of normal dogs but accelerates glycolysis in blood from diabetic dogs. Blood may have a phosphatase that can convert EAPP to TPP, which is decreased in diabetics, thus accounting for this stimulatory activity. Brin *et al.* (81) observed that red blood cells from thiamine-deficient rats have a 30 per cent decreased respiration and a 90 per cent decrease in production of $C^{14}O_2$ from glucose-2-C^{14}, with a threefold increase in pentose accumulation, which suggests that the deficient state results in an impairment in the hexose monophosphate cycle at the transketolase stage. All the changes could be reversed by injection of thiamine *in vivo*. Addition of thiamine *in vitro* reversed the pentose accumulation with a corresponding increase in a 7-C sugar. Kondo *et al.* (82) observed that the thiamine concentration was greater in the salivary gland of dogs than in the excreted saliva. In the saliva of dogs, total thiamine averaged 3.9 μg. per 100 ml., no free thiamine being present. In the parotid glands of the same animals, there was 150 μg. total thiamine and 2.4 μg. of free thiamine per 100 gm. of tissue. The maxillary glands had 51.7 μg. total thiamine per 100 gm. of tissue. No evidence for destruction of thiamine by dog saliva could be obtained. In human saliva, total thiamine averaged 7.7 μg. per 100 ml., and free thiamine averaged 0.3 μg. per 100 ml. When human saliva was allowed to stand at room temperature for 15 min., 17 per cent of the thiamine was destroyed, and after 30 min., 37 per cent had disappeared. Boiling the saliva immediately after collection prevented this destruction. The saliva of patients with cancer or liver insufficiency had a decreased ability to destroy thiamine.

In a continuation of studies of the pathways of thiamine biosynthesis in *Neurospora*, Harris (83) showed that pyridoxine inhibits thiamine biosynthesis. The inhibition can be overcome in a competitive manner by the pyrimidine moiety of thiamine. Inhibition of pyridoxine is also relieved noncompetitively by thiamine and inefficiently and noncompetitively by thiazole. These observations, together with the fact that 4-deoxypyridoxine, a known antimetabolite of pyridoxine, acts identically with pyridoxine, rules out the possibility that inhibition is caused by one of the known metabolic

functions of vitamin B_6. They suggest rather that pyridoxine prevents the synthesis of thiamine by competing with pyrimidine for the enzyme that brings about the coupling of pyrimidine to thiazole. It is not known whether pyridoxine and 4-deoxypyridoxine produce their inhibition simply by competing for enzyme surface or whether they can actually couple with thiazole to produce a thiamine analogue. Fuerst *et al.* (84) observed growth inhibition in *Neurospora* by the adenine analogue, 4-aminopyrazolo(3,4,d)pyrimidine (4-APP). The inhibition was partially relieved by thiamine, pyridoxine, or adenine. These data suggest that 4-APP may interfere with the metabolism of both adenine and thiamine. Chiao & Peterson (85) have extended the previous studies of Rabinowitz & Snell (86, 87) on interrelation of thiamine and vitamin B_6 in the growth of *S. carlsbergensis*. This organism has been widely used for vitamin B_6 assay, but Rabinowitz & Snell (86) found that the assay of vitamin B_6 with the organism is successful only if enough thiamine is added to the medium. If thiamine is omitted from the medium, *S. carlsbergensis* grows very well without vitamin B_6. Under these conditions, growth is inhibited almost completely by small amounts of thiamine, and this inhibition is alleviated by the addition of vitamin B_6. Chiao & Peterson (85) now observe that pantothenic acid and riboflavin can also relieve thiamine inhibition, though not so efficiently as pyridoxal. The relation between pyridoxal and thiamine is noncompetitive, i.e., 0.1 μg. of pyridoxal brought about approximately the same amount of growth in the presence of 0.1 to 10 μg. of thiamine. It was also shown that thiamine does not interfere with the synthesis of vitamin B_6, since even with thiamine-inhibited cells the amount of B_6 recovered was about 40 times as much as was added. If growth was allowed to occur in the absence of thiamine for about 13 hr., the subsequent addition of thiamine no longer produced an in hibition. Under anaerobic conditions both thiamine and pyridoxal are required for growth, but in much higher concentration. Under aerobic conditions, 0.01 μg. of thiamine and 0.1 μg. of pyridoxal gave maximum yield of yeast, whereas under anaerobic conditions 10 μg. of thiamine and 1.0 μg. of pyridoxal are required for the maximum response. The explanation for these interrelated growth phenomena is not yet at hand, but it seems clear that *S. carlsbergensis* can synthesize both thiamine and pyridoxal and grows rather well when there is no external supply of either. The trouble that occurs when thiamine is added and the relief on further addition of pyridoxal suggest that competition may occur at a point where the two vitamins are involved with a common metabolic substrate, such as pyruvate. This is further suggested by the partial relief of thiamine inhibition afforded by pantothenic acid and riboflavin, and their involvement in pyruvate metabolism.

Since both pyrithiamine and oxythiamine produce thiamine deficiency in animals but only pyrithiamine produces the typical neurological symptoms without reducing liver TPP or blood pyruvate levels, Woolley (88, 89) postulated a new metabolic function for thiamine indispensable to nervous tissue

and not related to TPP. Oxythiamine, on the other hand, increases both urinary excretion of thiamine and blood pyruvate in the rat, but fails to produce the neuromuscular syndrome (90). Two groups of workers have now presented observations that raise some question concerning the validity of Woolley's interpretation (91, 92, 93).

Koedam *et al.* (91) found that the TPP levels of pigeon brain, liver, heart muscle, breast muscle, and kidneys were much lower in the birds fed a thiamine-deficient diet plus pyrithiamine than in the controls that were simply thiamine deficient. They also found that the activity of pyruvic decarboxylase in breast muscle was markedly reduced after feeding pyrithiamine; but the loss of activity was not caused by destruction of the apoenzyme since full activity could be restored by adding TPP to the homogenates.

De Caro and his co-workers (92, 93) studied extensively the effects of pyrithamine and oxythiamine on the TPP content of tissues of mice and rats as well as the effects on thiamine excretion, body weight, and blood pyruvate. Examination of the liver, muscle, and brain tissue of these animals for both free thiamine and TPP revealed that most of the thiamine of the tissues exists as TPP, only a negligible fraction being free. Pyrithiamine caused a significantly greater decrease of TPP in all tissues than did either simple nutritional deficiency or oxythiamine administration. Oxythiamine, on the other hand, did not lower the thiamine content of the tissues over that caused by the dietary deficiency, except for liver. Urinary excretion of thiamine is increased 10 to 20 times by pyrithiamine and only 2.5 times by oxythiamine. Pyruvicaemia is markedly increased by both antagonists, but lasts after administration ceases only with pyrithiamine. Neurological symptoms of thiamine deficiency were present only in animals treated with pyrithiamine. However other findings cited by these authors indicated that, in B_1 avitaminosis of the rat obtained with a deficient diet, neurological symptoms were present only when the thiamine content of the brain was reduced to about 0.5 to 0.6 μg. per gm. Therefore it is the reduced thiamine content of the brain however obtained that causes the neurological symptoms. In the present experiment only the pyrithiamine-treated rats had brain thiamine levels of this order of magnitude. The authors conclude that pyrithiamine is a much more potent thiamine antagonist than oxythiamine. De Caro *et al.* (92) reported and extended Woolley's experiment (88) with mice. Here again only the administration of pyrithiamine led to the exhibition of neuromuscular symptoms; and the brain thiamine levels were markedly reduced. Oxythiamine administered under the same experimental conditions fails to produce, even in four times the dose of pyrithiamine, neuromuscular symptoms or any modification of blood pyruvate, body weight, or thiamine content of the brain and muscle beyond that seen with a dietary deficiency. Only in the liver did oxythiamine reduce the thiamine content. The authors conclude that the difference between oxythiamine and pyrithiamine lies in the fact that the latter is a more potent inhibitor of thiamine and produces neuro-

logical symptoms by reducing brain thiamine levels rather than because thiamine has a different function in nervous tissue.

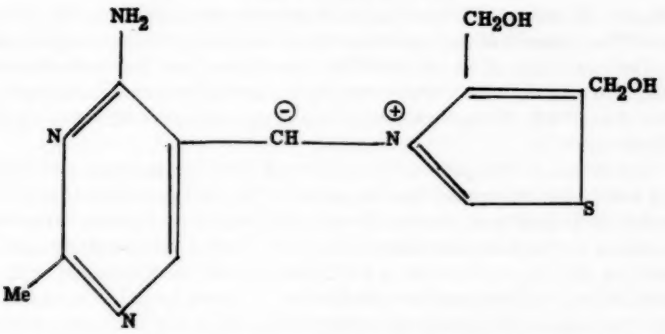
Navazio *et al.* (94) reported the preparation of pure crystalline oxythiamine phosphoric esters. The mono-, di-, and triphosphate esters of oxythiamine were prepared by the method of Viscontini *et al.* (95) and separated by cellulose column electrophoresis. It was found that, like thiamine, the ultraviolet spectra of the phosphate esters of oxythiamine are the same, but unlike thiamine esters, the spectra do not vary with pH. The phosphoric esters of oxythiamine are much more stable than the esters of thiamine. With the crystalline compound, they confirmed previous observations (96) of the inhibitory effect of oxythiamine phosphoric esters on the enzymes of pyruvate metabolism.

Through the synthesis of a number of phosphate esters of thiamine and a study of their properties, Bartos (97) attempted to explain the results of Siliprandi & Siliprandi (98), who reported that the chemical synthesis of thiamine triphosphate did not yield a pure compound but was contaminated with thiamine di- and monophosphate. Velluz *et al.* (99) had previously reported that chemically synthesized thiamine triphosphate had 80 per cent of the activity of cocarboxylase with yeast carboxylase. Siliprandi & Siliprandi (98), however, purified thiamine triphosphate by paper chromatography and reported that it had only 45 per cent of the activity of cocarboxylase. Bartos (97) synthesized the ortho- and pyrophosphates (groups on the nitrogen of the thiazole moiety) of thiamine ortho-, pyro-, and triphosphates. From an analysis of the chemical properties of these compounds, together with the foregoing observations from the literature, Bartos suggests the existence in yeast of two distinct apoenzymes, one combining with cocarboxylase and the other with thiamine triphosphate. Kiessling (100) has apparently provided the explanation of these controversial reports. He prepared thiamine triphosphate chemically and enzymically by incubating yeast with thiamine and pyrophosphate. The two products were extensively purified by three repeated chromatographic steps with paper. The two compounds were found to be hydrolyzed by potato apyrase at the same rate as ATP, suggesting a similar arrangement of phosphate groups. The enzymically synthesized compound was hydrolyzed in the same way, but somewhat more slowly possibly, owing to a contamination obtained from yeast during the isolation of the compound. Neither the chemically synthesized nor the enzymically synthesized thiamine triphosphate could replace cocarboxylase in a purified pyruvate decarboxylation system. But when the compounds were preincubated with potato apyrase at 0°C., which results in removal of the terminal phosphate group, both compounds exhibit TPP activity. These observations suggest that the positive and variable results of previous workers who measured the coenzymatic activity of thiamine triphosphate with a washed yeast preparation (not purified) were caused by the enzymatic formation of TPP from thiamine triphosphate in the crude preparation.

Marten (101) reported the occurrence of thiamine disulfide in urine. The intravenous injection of thiamine disulfide results in its immediate equilibrium with thiamine. Thirty min. after injection of thiamine disulfide, thiamine, thiamine orthophosphate, and thiamine disulfide were shown to be present in urine by paper chromatography.

It was reported by Ulbricht & Gots (102) that 4-amino-5-hydroxymethyl-2-methylpyrimidine, which is an inhibitory antagonist of the pyrimidine moiety of thiamine (103), will support the growth of pyrimidine-requiring mutant of *E. coli* if the thiazole moiety is simultaneously supplied. This observation suggested that the antagonist might be converted to an active thiamine-like compound. Accordingly, the thiamine analogue of the antagonist was synthesized and, contrary to expectations, was found to be a competitive inhibitor of thiamine. Shulman (104) observed that low doses (10 mg./kg.) of Daptazole (2, 4-diamino-5-phenylthiazole·HCl) together with the pyrimidine moiety of thiamine (2.5 mg. per kg.), could prevent symptoms of thiamine avitaminosis in rats on a thiamine-deficient diet. Larger doses of Daptazole (50 mg. per kg.) and pyrimidine (12 mg. per kg.) together, but not when administered alone, appeared to accelerate the onset of symptoms of thiamine deficiency. Thus Daptazole may enter thiamine metabolism, which might help to account for the wide range of physiological activity produced by this drug.

It had previously been suggested by Mizuhara (105) that the lone pair of electrons on the thiazole nitrogen of thiamine pseudobase acts as the active center for the catalysis of acetoin formation from pyruvate and for the diacetylmutase reaction. Since Karrer (106) suggested that the thiazole ring of thiamine may open to form thiamine thiol, which reacts to form a thioacetal of pyruvate with subsequent decarboxylation, and Matsukawa *et al.* (107) have shown that one of the acetyl groups attached to the thiazole sulfur in chemically prepared O,S-diacetylthiamine can serve as an acetyl donor, Mizuhara and his co-workers have now tested a number of thioacetals and thiol compounds for their ability to catalyze acyloin condensation in the presence of pyruvate, but in the absence of thiamine (108). Cysteine, thioglycolic acid, thioglycolid-pyruvic acid addition compound, thiophenol, and O,S-diacetylthiamine were all unable to catalyze acyloin condensation. Thus it would appear that thiamine is a rather specific requirement for acyloin condensation under these specific conditions. Breslow (109) used the nonenzymatic test of Mizuhara, in which pyruvate and acetaldehyde undergo acyloin condensation when incubated at pH 8.4, to test a variety of substituted thiamine derivatives for their ability to catalyze acyloin condensation. The reaction was catalyzed by thiamine, N-methylthiamine·HCl, and N-benzyl-4-methylthiazolium·HCl, but oxythiamine, N-ethyl-4-methylthiazolium·Br, and 4-amino-5-hydroxymethyl-2-pyrimidine were all inactive. Because of these reactivities, Breslow proposes a mechanism for the decarboxylation of pyruvate that involves the addition of pyruvate to the active methylene group of an *ylid* of the structure shown in Formula 1.



Then by appropriate electronic rearrangements the decarboxylation should occur easily. Wiesner & Valenta (110) proposed a theoretical scheme to account for the reactions of thiamine pyrophosphate, which also involved an *ylid*. In this scheme the reaction is visualized as taking place on the amino group of the pyrimidine moiety to form a Schiff base, and then abstraction of a proton from the methylene carbon to produce a similar *ylid*. Ingrahm & Westheimer (111) presented evidence that excludes the participation of an *ylid* in the decarboxylation of pyruvate by showing that thiamine does not take up deuterium when the pyruvate decarboxylation is carried out in D_2O as solvent. Thus the status of the exact mechanism involved in TPP-catalyzed reactions still awaits the successful isolation and characterization of an intermediate compound.

α-Lipoic Acid (Thioctic Acid)

A new synthesis of 6:8-thioctic acid has been reported by Braude *et al.* (112). Starting with hept-6-enoic acid, a 5-step synthesis gives an over-all yield of 20 to 30 per cent, which is not so good or so convenient as the synthesis devised by Reed and Niu (113). Two groups (114, 115) have independently synthesized S^{35}-α-lipoic acid. Thomas & Reed (115) obtained a crystalline product containing 68 μc. per mg. After the material had been stored in dry, solid form in the refrigerator for 10 weeks, a radiation decomposition of about 10 per cent was observed. Storage of dilute solutions (1 mg. per ml.) of S^{35}-α-lipoic acid in 95 per cent ethanol or in 1 per cent aqueous sodium bicarbonate in a refrigerator caused no radiation decomposition during the 10-week period. Adams (114) also successfully prepared crystalline S^{35}-α-lipoic acid of about 1 μc. per mg., but attempts to prepare highly active material of the order of 200 μc. per mg. failed to yield a crystalline product. A study of the sensitivity of thioctic acid to Co^{60} γ rays indicated that material of specific activity of 200 μc. per mg. would be decomposed at the rate of 0.3 per cent per day, which is in rather good agreement with the figures reported by Thomas & Reed. The radiation damage to

α-lipoic acid however is insufficient to account for their failure to obtain a crystalline material of high specific activity and is at present unexplained.

The synthesis of *dl*-dimethyldihydro-α-lipoic acid has been described (116). No α-lipoic acid activity was shown in the enzymatic assay of Gunsalus *et al.* (117). Demethylation of the compound gave only low yields of α-lipoic acid.

The details of the previously announced (118) synthesis of (+)-α-lipoic acid and (−)-α-lipoic acid have appeared (119). *dl*-3-Acetylthio-7-carbethyoxyheptanoic acid was resolved into its (+) and (−) isomers. These were converted to the acid chloride, reduced with sodium borohydride and subjected to alkaline hydrolysis to yield 8-hydroxy-6-thiol octanoic acid. Replacement of the hydroxyl with sulfhydryl followed by oxidation gave (+) or (−) α-lipoic acid, depending on the configuration of the starting material. In the enzymatic assay (117) (+)-α-lipoic acid was shown to have twice the activity of the *dl* mixture and (−)-α-lipoic acid was essentially inactive. The configuration of the optical isomers has been determined by Mislow & Meluch (120). Wagner *et al.* (121) reported a number of observations on the stability and other properties of α-lipoic acid. Of practical interest are studies on the thermal stability under a variety of conditions of pH and degree of admission of oxygen. These should be of considerable value in devising methods for the extraction from and estimation of α-lipoic acid in biological materials. For example, autoclaving, in 0.1 or 2 *N* NaOH in open test tubes at 15 lb. pressure for 3 hr. destroyed 80 per cent of enzymatic activity. If, however, autoclaving is carried out under an atmosphere of nitrogen in sealed tubes, the activity is quantitatively recovered from 0.1 *N* alkali and about 80 per cent recovered from 2 *N* alkali. The same authors report the conversion of DL-α-lipoic acid to DL-α-lipoamide and DL-α-lipol, through the intermediate acid chloride.

Gunsalus *et al.* (122, 123) reported in detail their work on the biosynthesis of lipoic acid derivatives. An enzyme, a thioestertransacetylase, transports one acetyl group from acetyl-S-CoA to reduced lipoic acid (dihydrolipoic acid). The enzyme shows optical specificity for (−)-dihydrolipoic acid, which is prepared from the dextrorotatory isomer of (+)-α-lipoic acid, by reduction with sodium borohydride. Mono-S-acetyl-dihydrolipoic acid was prepared chemically from its methyl ether by reaction with acetic anhydride and then removal of the methyl groups by acid hydrolysis, and biologically from acetyl phosphate, CoA, phosphotransacetylase, and dihydrolipoic transacetylase. The properties of the two compounds revealed that chemical acetylation results in the formation of 8-S-acetyl,6,8-dithioloctanoic acid whereas the product of enzymatic transacylation is 6-S-acetyl,6,8-dithioloctanoic acid. Thus the chemical reagent preferentially acylates the primary —SH group whereas the enzymatic reaction results in acylation of the secondary —SH group. It is interesting that, although dihydrolipoic transacetylase shows optical specificity for (−)-dihydrolipoic acid, dihyro-

lipoic dehydrogenase oxidizes both optical isomers. No report is given on any possible enzymatic activity of chemically synthesized 8-acetyl,6,8-dithiooctanoic acid. Reed and his associates (124, 125, 126), working with cell-free extracts of lipoic acid-deficient *Streptococcus faecalis*, showed that pyruvate and α-ketobutyrate dismutation by such extracts is greatly activated by preincubation of the extract with lipoic acid. With S^{35}-labeled lipoic acid, a direct relation between enzymatic activation and uptake of lipoic acid into a nondialyzable form was observed. The extract was resolved into two fractions, one of which appeared to be involved in the enzymatic binding of lipoic acid. Magnesium ion, phosphate, and ATP seem to be necessary for the conversion of lipoic acid to a bound form.

Bornstein & Hartman (127) reported that addition of lipoic acid to liver slices increased the production of labeled CO_2 from uniformly labeled glucose, had no effect on the yield of CO_2 from 2-labeled pyruvate, and decreased the yield from 1-labeled acetate, suggesting that the addition of thioctic acid facilitiates the oxidative decarboxylation of pyruvate.

Cutulo (128) described the preparation of a DPN dependent lipoic acid dehydrogenase from yeast. The same preparation is less active in the dehydrogenation of cysteine. In a continuation of their studies on the mechanism of photosynthesis, the Calvin group (129) performed kinetic experiments of the photosynthetic and respiratory intermediates when steady-state photosynthesis is changed from light to dark and back to light again. The significance of this work with respect to lipoic acid lies in the observation that turning off the light results in a transfer of radiocarbon from phosphoglyceric acid to citric and glutamic acids. When the light is turned on again, radioactive carbon decreases in citric acid. These data are interpreted to indicate a light-induced inhibition of the Krebs' cycle and are considered to be futher evidence in support of the theory (130) that light inhibits the oxidation of pyruvate to acetylCoA and CO_2 by a reduction of lipoic acid.

In a study of the induction of plaque formation by ultraviolet irradiation of a lysogenic strain of *E. coli*, Borek *et al.* (131) observed that acetate can inhibit the ultraviolet induced plaque formation, i.e., it increases the number of cells that survive as colony formers. CoASH had no effect alone, but potentiated the effect of acetate. Pyruvate plus lipoic acid was more effective than acetate and CoA. Lipoic, by itself, was the most potent single adjuvent for the restoration effect. In this connection, Spizizen (132) observed that coincident with infection of *E. coli* by phage T2 or T7, a lesion appears in pyruvate metabolism. He has further shown (133) that part of the lesion is caused by leakage from the infected cells of certain coenzymes of pyruvate metabolism, such as TPP, CoA, and DPN. These observations, together with the above effect of lipoic acid suggest that pyruvate metabolism may play an important role in controlling phage growth.

Fischer (134) observed that mitochondria prepared from livers of rats with ethionine-induced lesion have a reduced content of lipoic acid. This

observation is coupled with an increase in blood pyruvate in the ethionine-treated animals and a reduced pyruvate oxidation by mitochondria prepared from such animals. The *in vitro* and *in vivo* addition of lipoic acid to the mitochondria failed to restore pyruvate oxidation to normal levels. There was undiminished urinary excretion of lipoic acid and a normal kidney lipoic acid content in such animals, which led the authors to the conclusion that in the ethionine-treated animals, total-body synthesis of lipoic acid is normal, but hepatic binding of lipoic acid is impaired.

Fridovich & Handler (135) reported that an enzyme preparation from dog liver that catalyzes the oxidation of sulfite will also oxidize cysteine and dimercaptolipoic acid. They postulate a reaction mechanism that involves the addition of the bisulfite ion across a disulfide compound on the enzyme surface to form an intermediate hemithiosulfonate complex. This complex is then hydrolyzed to form sulfate and a dimercaptan. The oxidation process would result in the regeneration of the disulfide from the dimercaptan. Because of the rapid oxidation of reduced lipoic acid by the enzyme, they postulated that lipoic acid may be the disulfide on the enzyme surface. Since the chemical synthesis of lipoate hemisulfonate was unsuccessful, this hypothesis could not be tested directly. It is interesting that Sorbo (136) has postulated a mechanism for the action of rhodanese that similarly involves the addition of thiosulfate across an enzyme-bound disulfide, followed by hydrocyanolysis of the thiosulfate addition product. Now Cutulo & Reduzzi (137) report that of a series of thiol-containing compounds, reduced lipoic acid gives the best protection against KCN poisoning in mice. Cysteine, pantetheine, and thiosulfate afforded little protection. The authors suggest that lipoic acid may function as a coenzyme in rhodanese.

Several groups of workers (138, 139, 140) failed to confirm the report by DeBusk & Williams (141) that minute amounts of lipoic acid incorporated in purified diets increased the growth rate and feed utilization of White Leghorn chicks and rats. Stokstad *et al.* (138) made a careful study with chicks and even obtained a sample of the diet used by DeBusk and Williams without observing an influence on growth rate. They also demonstrated that the developing chick embryo is capable of synthesizing lipoic acid. Because Olson & Dinning (142) reported that a sulfur-deficient diet resulted in a decreased level of liver CoA, the influence of a methionine-deficient diet on the lipoic acid content of chick liver and heart was studied. Although the diet was sufficiently deficient in methionine to restrict growth severely there was no effect on the lipoic acid levels of the heart and liver. Supplee *et al.* (140) attempted to determine whether discrepancies between results in various laboratories on the effect of lipoic acid on chick growth might be caused by differences in chicks used in the study. They failed to observe an effect of lipoic acid on the growth rate of three different genetic strains of chicks and suggested that the differences could be caused by an indirect effect of lipoic acid through action on certain microorganisms in the intestinal tract, which might differ in the various laboratories.

Riboflavin

Beal (66) studied the riboflavin intake in children up to 5 years of age. In general, riboflavin intake shows a rise during the first year and gradually decreases during the second and third year as the milk consumption decreases. Bartlett (143) was unable to find any correlation between riboflavin intake and total riboflavin in the plasma of these children. Bessey *et al.* (144) concluded that red blood riboflavin determinations are the most reliable index of nutritional intake of the vitamin. They found that when human subjects were restricted to 0.50 to 0.55 mg. of riboflavin per day, the red cell riboflavin was 10.0 to 13.1 μg. per 100 ml. and 20.2 to 27.6 μg. per 100 ml. in control groups that received 2.55 to 3.55 mg. of riboflavin per day. In the plasma, free riboflavin plus FMN reflected dietary intake, but Bessey *et al.* found several glaring exceptions that tend to make these measurements a less reliable index of dietary intake. The plasma FAD riboflavin and white blood cell riboflavin were not significantly altered by dietary restriction of the vitamin. Burch *et al.* (145, 146) measured changes in FMN, FAD, and certain flavin enzymes in the tissues of riboflavin-deficient rats. The amounts of FAD, FMN, and flavin enzymes in the brain are apparently unaffected by riboflavin deficiency. The changes in the liver were most marked. In general, FMN is lost from the tissues more rapidly than FAD, and FAD is regenerated more rapidly than FMN when riboflavin is injected. Of the flavin enzymes measured, DPNH dehydrogenase had the highest activity in all tissues and showed no decrease in moderate riboflavin deficiency. In severe deficiency it showed a downward trend. In the liver, the enzymes examined showed the following order of sensitivity to riboflavin deficiency: glycolate oxidase, D-amino acid oxidase, glycine oxidase, xanthine oxidase, L-amino acid oxidase, and DPNH dehydrogenase. Xanthine oxidase decrease was parallel to the decrease of FAD, and the decrease of D-amino acid oxidase and glycolic acid oxidase was parallel with the decrease in FMN. It is interesting that with the latter two enzymes, both the protein and the coenzyme moieties disappear in depleted rats, suggesting that riboflavin may play a special role in either the synthesis or the maintenance of these enzymes. Modi & Owen (147) characterized the various forms of riboflavin in the milk of several species by using paper chromatography and paper electrophoresis. With the milk of cows, goats, ewes, and rabbits the bulk of the riboflavin was free, with a small amount of FAD appearing. In human, mare's, and sow's milk, most of the riboflavin was present as FAD with little or none in the free form.

A riboflavin-deficient diet given to female rats during gestation has little effect on fetal development (148). If, however, the vitamin deficiency is accentuated by the addition of galactoflavin for the entire gestation period or for only 4 to 6 days early in gestation, there is a high incidence of fetal death and congenital abnormality. Skeletal defects predominate when the antimetabolite is given throughout gestation. Cardiovascular defects were the only abnormalities observed from a transitory deficiency induced by galacto-

flavin ingestion from days 7 to 11. The abnormalities induced by the antimetabolite are completely prevented when riboflavin is included in the diet.

Morgan and her co-workers (47, 48, 149) continued their studies on the effect of stress in riboflavin-deficient animals in an attempt to explain the breakdown of the pituitary-adrenal cycle in this deficiency. The stress of anoxic anoxia failed to produce changes in ascorbic acid content of riboflavin-deficient rats. Yet the adrenals of such deficient animals were shown to be capable of normal response to ACTH, and the pituitary glands of the animals appeared to be normal since regeneration of enucleated adrenals proceeded normally. The authors suggest that the breakdown of the pituitary-adrenal cycle in riboflavin-deficient animals may lie in the mechanism by which the pituitary is stimulated to secrete ACTH.

Egami *et al.* (150, 151) demonstrated that flavin monosulfate inhibits D-amino acid oxidase and acts as a competitive inhibitor of riboflavin in the growth of *L. casei* and *S. faecalis*. The inhibition indices with FAD, FMN, and riboflavin were 5, 6, and 63, respectively.

Yagi (152) described in detail a simplified lumiflavin method for the microdetermination of flavin compounds in tissues. Since the method does not distinguish between the various forms of riboflavin, these are first separated by paper chromatography and paper electrophoresis (153). Yagi *et al.* (154) described the preparation of FAD of 85 per cent purity from *Eremothecium ashbyii* by partition between phenol and water, counter-current distribution, and "Florisil" adsorption chromatography in about 50 per cent yield. De Luca *et al.* (155) made use of a previous observation of Horecker (156) indicating that D-amino acid oxidase could be measured spectrophotometrically by coupling to the lactic dehydrogenase system to set up a highly specific, rapid quantitative assay for FAD. A comparison of the new method with the fluorimetric method (157) indicated reasonable agreement in most cases except in situations where flavin dinucleotides not identical with FAD may be present. In the latter case, the hydrolysis with trichloroacetic acid would lead to FMN and, accordingly to higher values. Huennekens & Kilgour (158) synthesized FAD by condensing adenylic acid with FMN by means of di-*p*-tolylcarbodiimide, as described by Kohrana (159). They observed a certain amount of cyclic FMN as a principal product and, when the more reactive dicyclohexylcarbodiimide was used, cyclic FMN was the sole product. They prepared flavin inosine dinucleotide and found it inactive with D-amino acid oxidase. De Luca & Kaplan (160), utilizing trifluoroacetic acid as condensing agent, synthesized FAD from AMP and FMN. The product is purified by the method of Whitby (161). Using this method, they prepared a number of analogues of FAD in which the adenine moiety was replaced with guanine, cytosine, and uracil. Another analogue was prepared by coupling FMN with nicotinamide mononucleotide. In each case, the analogues were biologically inactive either as activators or inhibitors in the D-amino acid oxidase system.

The interesting problem of the biosynthesis of riboflavin continues to

engage the attention of several groups of workers. Plaut (162) has succinctly reviewed the current status of information about the origin of the various carbon atoms and presented some new data on the origin of the ribityl side chain (163). Using methyl- and carboxyl-labeled acetate and uniformly labeled glucose, glucose 1-C^{14}, glucose 6-C^{14}, and glucose 2-C^{14} (163), he found that $C^{14}H_3COOH$ labels C_5' and C_2' to C_4' of the ribityl side chain. Glucose 1-C^{14} labels C_1' and C_5' predominantly, with little label appearing in C_2' to C_4'. Glucose 6-C^{14} labels C_5' almost exclusively with some label appearing in C_1' whereas glucose 2-C^{14} puts a high proportion of label in C_2' to C_4', about half as much in C_1', and relatively little in C_5'. From this pattern of labeling, it seems clear that the hexosemonophosphate pathway is responsible at least in part for the formation of the ribityl side chain, but other pathways must also play an important role in the biosynthesis of the ribityl moiety. In this connection, Stadtman (164) pointed out that conclusions drawn from experiments in which labeled glucose or other large molecules are used with one microorganism may not be generally applicable to all instances of riboflavin biosynthesis, since such results will depend in large measure on the pathways of glucose metabolism in each organism. Goodwin & Jones (165) studied the incorporation of C^{14}-serine into riboflavin in an attempt to explain the phenomenon that although glycine is incorporated into riboflavin (166), it does not specifically stimulate flavin synthesis in *E. ashbyii*, whereas serine does stimulate flavin synthesis and the stimulatory effect of serine and purines are additive. They found that uniformly labeled serine is incorporated into riboflavin to the extent of 4 to 7 per cent and the label is confined to positions 2, 4a, and 9a. Serine 2-C^{14} is incorporated almost exclusively into position 2. The labeling pattern is consistent with the idea that serine is converted to glycine and formate before incorporation into riboflavin. It is suggested that the reason why serine, but not glycine or glycine plus formate, is stimulatory for riboflavin synthesis is that serine is more easily converted to active glycine and active formate. Adenine, added with labeled serine, almost completely diluted out the serine label in riboflavin, which is consistent with the assumption that serine is being incorporated via an adenine-like pool. McNutt (167) demonstrated that the pyrimidine ring of adenine is incorporated *in toto* into the isoalloxazine ring of riboflavin, thus accounting for the previous observation that C_8 of adenine is lost during its incorporation into riboflavin (168) and explains the labeling with CO_2, formate, and glycine. The failure of pyrimidines such as uracil or thymine to stimulate production of riboflavin by *E. ashbyii* (169) whereas adenine does, suggests that the pyrimidine moiety of adenine may be activated before its incorporation into riboflavin. Another approach to the problem of riboflavin biosynthesis is being used by Stadtman (164). Using the soil-enrichment culture technique to select microorganisms that can utilize riboflavin as their sole source of carbon, nitrogen, and energy under anaerobic conditions where one might expect the accumulation of degradation products that may be intermediates in the biosynthesis, Miles & Stadt-

man (170) isolated an organism that ferments riboflavin and accumulates a number of flavin derivatives during growth. One of these products was identified as 6,7,-dimethyl-9-(2′-hydroxyethyl)-isoallaxozine. Yanagita & Foster (171) described the preparation of a cell-free, particle-bound enzyme from *Pseudomonas riboflavina* that hydrolyzes riboflavin to lumichrome and ribitol. The enzyme appears to be adaptive, since its formation depends on a high concentration of riboflavin in the culture medium. With a new temperature-independent, riboflavin-requiring mutant of *Neurospora crassa*, Garnjobst & Tatum (172) were unable to demonstrate the accumulation of any precursors of riboflavin. It is known that the optimum iron concentration for the production of riboflavin by *Candida guilliermondia* is 5 to 10 μg. per l.; higher iron concentrations lead to sharply reduced yields of riboflavin (173). Enari (174) now reports that cobalt in a concentration of $10^{-4}M$ (5.9 mg. per l.) greatly enhances riboflavin production and shifts the optimum iron concentration to 560 μg. per l., which in cobalt-free medium inhibited the production of riboflavin.

It had been found that there was less FAD in the livers of alloxan diabetic rats than in normal animals (175). Siliprandi *et al.* (176) have now demonstrated that this is caused by decreased ability of liver cells from diabetic animals to carry out the synthesis of FAD from FMN plus ATP. Insulin did not increase significantly the synthesis of FAD by the isolated liver cells.

In an attempt to explain why high levels of riboflavin prevents the depletion of hepatic riboflavin in animals fed 2-acetylaminofluorene without having any effect on the carcinogenic properties of this drug, Wase (177) measured the half life of riboflavin-C^{14} in the liver of normal rats and rats fed 2-acetylaminofluorene. In normal rats the half-life of riboflavin is 6.53 ± 0.5 days, which is reduced to 1.09 ± 0.19 days by the feeding of 2-acetylaminofluorene. Thus it would appear that the latter compound increases the metabolic turnover of hepatic riboflavin, which would lead to a state of depletion unless an increased daily supply were made available.

By replacing the ribityl side chain of riboflavin with a $-CH_2 \cdot CHO$ group or a hydroxyethyl group, Fall & Petering (178) synthesized a number of analogues of riboflavin that appear to be competitive inhibitors of riboflavin in microorganisms and in the rat. It is interesting that the hydroxyethyl derivative has antifungal and antibacterial activity, since it is indentical with the product isolated by Miles & Stadtman (170) from an anaerobic fermentation of riboflavin.

Theorell and his co-workers continued their interesting studies of the effect of various agents on the association (k_1) and dissociation (k_2) velocity constants between FMN and OYE (179). The techniques used and some of the early data were reported in this volume last year (46). They modified OYE by acetylating amino groups and by iodinating tyrosine and measuring the effect of these modifications on the velocity constants. Acetylation of half the amino groups led to a decrease in k_1 from 1.6 times 10^6 to 0.14 times 10^6 and a strong increase in k_2, which supports their earlier conclusion that

amino groups in juxtaposition to the phosphate group may serve as a binding site for FMN. In spite of this strong reduction of k_1, the binding capacity of OYE of 1 mole of FMN per mole of enzyme remained unchanged. Because riboflavin also combines with the enzyme, it would appear that the amino groups are not essential for the protein-isoalloxazine linkages that quench the fluorescence, but rather that the amino groups serve to stabilize the complex. OYE was found to be free of sulfhydryl groups, and thus iodination was considered to affect only tyrosyl residues. Such treatment of OYE, in which six of the 24 tyrosine groups were iodinated, led to a decrease in k_1 from 1.4 times 10^6 to 0.06 times 10^6 and a strong increase in k_2. This observation was interpreted to indicate that the tyrosine residue is responsible for the binding of the riboflavin part of the molecule. FAD, riboflavin, and a compound that appears to be riboflavin diphosphate are all more loosely bound to OYE than FMN, suggesting that the specific binding of FMN is the result of complementariness of the combining region on the protein molecule. Vestling (180) performed a reductive titration of highly purified OYE with reduced safranine T and potassium indigo disulfonate as mediator and obtained an E_0' value at pH 7 of -0.123 volt. From this value, he calculated K_r of $6\times10^{-11}M$, which indicates the extremely tight binding of $FMNH_2$ to its apoenzyme.

Lowe & Clark (181) presented potentiometric titration curves for FAD and FMN from which a value of $E_0=0.187$ volt for FMN was obtained and a calculated value of E_m at pH 7.0 -0.219 volt for FAD. The data indicate that the oxidation-reduction over the pH range 0.89 to 10.9 is a reversible process that involves the formation of a semiquinone as an intermediate. At constant pH, the slopes of the titration curves for FMN increase with increasing concentrations of FMN, which indicates the formation of a dimer. Beinert (182, 183), in an elegant series of studies, obtained convincing spectral evidence that in the reduction of flavins the compounds pass through semiquinoid intermediates having distinct spectra. The oxidation of fully reduced FMN and FAD was recorded continuously with a recording spectrophotometer. Absorption bands were observed that were maximally developed at 50 per cent oxidation and were considered to be caused by semiquinoid intermediates in their monomeric and dimeric forms. The monomeric form is characterized by an absorption maximum of 565 mμ at pH 2 to 7. The principal absorption of the dimeric forms occurs between 700 and 1100 mμ. It is significant that the molar absorption in this region is considerably increased when the concentration of FMN is increased from 2 times 10^{-4} to 4 times 10^{-3} M, suggesting that dimerization of the free radicals increases with increasing concentration. These studies were extended to flavoproteins, where it was observed that reduction of the fatty acid acyl-CoA dehydrogenase, which is a yellow flavoprotein, with dithionite led to the appearance of a new absorption band between 500 and 650 mμ while the absorption at 450 mμ is decreased. The appearance of the new band is a transient phenomenon that appears only during the few seconds in which the

flavin absorption at 450 mμ fades from its maximum to its minimum. The band also appears briefly upon reoxidation of the reduced enzyme. The intermediate responsible for the new absorption band is considered to be a free radical. The amount of the intermediate that forms from enzyme-bound FAD is about 20 times as great as that formed from free FAD. The intermediate is stable for hours when it is produced during the oxidation of substrate by the enzyme, but lasts only a few seconds when it is produced by dithionite. This suggests that the substrate can stabilize the free radical in some manner. Singer & Kearney (184, 185) reported the isolation of several flavin peptides from tryptic digestion of beef heart succinic dehydrogenase. One such flavin containing 3 amino end groups and 28 to 32 amino acids was fully as active as FAD in the D-amino acid oxidase test. Other fractions differing only slightly in composition showed little or no coenzymatic activity. Since a number of different FAD-containing peptides are obtained after digestion, these peptides may represent the points of attachment of FAD to the protein.

Evidence was presented by Dietrich & Harland (186) for a chemical reaction between hydroxylamine and riboflavin, FMN and FAD that results in a decrease in absorption at 245 mμ with riboflavin and FMN and at 240 mμ with FAD. The reaction is strongly pH dependent, starting at about pH 7.5 and increasing linearly with increasing pH to a maximum at pH 11. A similar change in spectrum is brought about by cyanide and is also pH dependent. Unlike the reaction with hydroxylamine, which is reversible by shifting the pH to the acid side, the cyanide reaction appears to be irreversible.

Inositol

Eagle *et al.* (187) demonstrated a nutritional requirement for *myo*-inositol by human cells growing in tissue culture. Of a total of 19 cell lines (17 human and two mouse), all but three show a requirement for *myo*-inositol. The 16 cultures, seven of which were derived from cancer tissue, nine from normal tissue, and three from bone marrow of patients with metastatic cancer, had remarkably consistent quantitative requirements for inositol, varying from 10^{-8} to 10^{-6} *M*. This concentration is higher than that for most vitamins and is of the same order of magnitude as the requirement for choline. Thus it is difficult to decide whether inositol is functioning here as a vitamin or as a metabolite. Nevertheless, this demonstration of the requirement for inositol by mammalian cells is highly significant in view of the equivocal evidence concerning the need for inositol by mammals. Thus Halliday & Anderson (188) isolated *myo*-inositol-C^{14} from the carcasses of rats that had received injections of glucose 1-C^{14}, confirming the previous finding by Daughaday *et al.* (189) of the synthesis of inositol from uniformly labeled glucose in the immature rat and in the chick embryo. The rate of synthesis of inositol by rat is very low; even after the labeled glucose had been in the body for 14 hr., the specific activity of the isolated inositol was only 3 per cent of that of the liver glycogen.

Magee *et al.* (190) found that the tranquilizing drugs, chlorpromazine (0.1 m*M*) and azacyclonol (1.0 m*M*) cause an increased uptake of P^{32} into inositol phosphate when added to respiring slices of guinea pig brain. The labelling with inorganic P^{32} was confined to inositol phosphate, glycerophosphate, and glycerolphosphorylserine with a decrease in labeling of glycerolphosphorylcholine and glycerolphosphorylethanolamine. The drugs had no effect on the P^{32} labeling of acid-soluble phosphates. Because of these observations, the authors conclude that the effect of chlorpromazine and azacyclonol *in vitro* is to increase the incorporation of inorganic P^{32} into diphosphoinositide and phosphatidylserine of brain slices.

Hutchison *et al.* (191) made an extensive study of the nonribonucleotide phosphate compounds previously observed as contaminants of the ribonucleotide fraction of Schmidt-Thannhauser digests of liver tissue. By ionophoresis and Dowex chromatography, a large number of phosphate-containing compounds were found. Many of the compounds were phosphopeptides, but inositol and some of its phosphate derivatives were also found. Acid supernatants prepared from liver, ox brain, bull sperm, and sea urchin eggs contained free inositol in the RNA fraction. Inositol monophosphate was identified in such extracts from liver and brain, but inositol diphosphate was observed only in brain extract. Acid supernatants prepared from different cell components and assayed for inositol by the *Neurospora* assay gave evidence for the occurrence of inositol in calf thymus nuclei, rat liver microsomes, mitochondria, and supernatant.

The phosphoinositide fractions from soy beans and ground nuts were isolated and hydrolyzed with acid, and the resulting inositol phosphates separated by ion-exchange chromatography (192). The inositol phosphatides of soy bean and ground nut appear to be identical. Inositoldiphosphate was not isolated and is presumed to be absent in these tissues. Among the hydrolysis products, inositolmonophosphate, linked glycosidically to galactose or arabinose, was identified. McKibben (193) isolated an apparently new sugar-free inositol glycerol phosphatide from a commercial whole lipide extract of horse liver. By a combination of procedures involving alcohol precipitation, chromatographic fractionation on a silicic acid column, and counter current distribution, an essentially homogeneous fraction was prepared containing inositol, phosphorus, glycerol, and fatty acid in the molecular ratio of 1:1:1:2. The substance is similar to the inositol glycerol phosphatidic acid isolated from wheat germ (194) and soy bean (195) except for differences in the fatty acid component. One interesting property of the substance is that it is six to ten times as active microbiologically as the soy bean inositide or phytate, which suggests that different isomers of parent inositides may exist in nature.

Using ion-exchange chromatography, Malangeau (196) prepared extracts rich in cyclohexitols from various animal tissues. Free inositol is removed by dialysis and the inositides are then separated by paper chromatography. With this technique, *myo*-inositol was found in muscle, kidney, liver, and egg yolk (197) in the free and lipide forms. Scyllitol was never

found in these tissues; however, examination of human urine with this technique revealed the presence of *myo*-inositol in every case and scyllitol was found in 67 per cent of the urines examined (198). About 35 to 85 mg. of total inositol is excreted per l. of urine.

Patrick *et al.* (199) describe the isolation of a monoamino analogue of inositol, inosamine, from hydrolysis of an antibiotic whose structure is not given. Previous inosamines, likewise isolated from antibiotics, were found to be diamines (200, 201). This represents the first description of a monoinosamine.

Desjobet & Petek (202) used paper chromatography to follow the progress of both acid and enzymatic hydrolysis of inositol hexaphosphate. By this technique they showed that the phosphoric ester isolated from wheat germ is a true inositol pentaphosphate. The chromatographic procedure did not allow the separation of two isomeric inositol monophosphates—one arising by acid hydrolysis and a different one produced by enzymatic hydrolysis. Arnold (203) similarly studied the products of acid hydrolysis of phytic acid separated by paper electrophoresis. A kinetic analysis of the data revealed that some of the isomers produced by acid hydrolysis are more resistant than others to further acid hydrolysis. The major difficulty of paper electrophoresis lies in its inability to separate hexa- and pentaphosphates.

De Robichon-Szulmajester (204) published details of work reported last year (46) showing that certain strains of *S. cerevisiae* that need pyrimidines for growth exhibit a requirement for inositol when grown on uracil or orotic acid but not when grown with cytosine, uridine, or uridylic acid. It is believed that inositol may function in the transformation of uracil to cytidine or uridine.

The isolation of the inositol dehydrogenase from *Aerobacter aerogenes* was described by Larner *et al.* (205). The properties of the enzyme were studied with a view toward developing an enzymatic method for inositol assay. The enzyme catalyzes a DPN-dependent dehydrogenation of *myo*-inositol, yielding a keto compound that may be *scyllo*-inosose. The equilibrium constant K_{H^+} is 3.8×10^{-3} *M*. Unfortunately, the association constant for *myo*-inositol at pH 9.0 is 1.25×10^{-3} *M* and the reverse reaction is favored, which does not permit the use of the spectophotometric assay for the determination of inositol. Inositol dehydrogenase activity, however, can be coupled to ferricyanide as an electron acceptor. With this method oxidation of *myo*-inositol, up to 0.4 μM gave quantitative oxidation, suggesting that this system may be developed into a suitable assay system.

Darbre & Norris (206) followed the changes in free and combined inositol during the germination of oats. Total inositol was measured microbiologically (207) after acid hydrolysis, and free inositol was measured similarly after extraction with dilute HCl. In general, during the germination process there is a net decrease of about 10 per cent in total inositol. The free inositol content increases by 100 to 200 per cent. It would appear from these results

that inositol is liberated from its combined form during germination and some of the resulting free inositol is destroyed during the process.

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THE FAT-SOLUBLE VITAMINS[1,2,3]

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Vitamin A

Chemistry, analysis, and assay.—Carotenoids are not discussed in detail. Isler *et al.* (1 to 6) and Karrer *et al.* (6a) give details of technical (1) and new (2) syntheses of β-carotene, new methods of chain lengthening and rearrangements of double bonds in the synthesis of ring components (3), the syntheses of 3:4,3′:4′-*bis*-dehydro-β-carotene and 3:4-mono-dehydro-β-carotene (4), of iso-zeaxanthin (5), lycopene (6), 6:7,6′:7′-tetra-hydrolycopene (6a), and three new carotenoids (2).

3:4′,3′:4′-*bis*-Dehydro-β-carotene stands in the same relationship to vitamin A_2 (dehydro vitamin A) as does β-carotene to vitamin A_1, and a number of 3:4-dehydrogenated systems would thus be expected to have biological activity. Isler *et al.* (5) have shown that 3:4,3′:4′-*bis*-dehydro-β-carotene has 38 per cent and 3:4-monodehydro-β-carotene 75 per cent of the biological activity of β-carotene (3). Similarly, deoxylutein I, now shown by Zechmeister *et al.* (7) to be 3-hydroxy-3:4-dehydro-β-carotene, although inactive in previous tests (8), has since been found in rat growth tests to have a biological activity 10 per cent that of β-carotene (7). Zechmeister (9) in studies of the reaction of carotenoids with boron trifluoride has found a new isomer of vitamin A_1, probably 4-hydroxy-axeropthene.

The structure attributed to echinenone, the pro-vitamin A from the gonads of the sea urchin, *Strongylocentrotus lividus*, by Goodwin & Taha (10), 4-keto-β-carotene, is correct (11, 12). Canthaxanthin has been found to be 4:4′-diketo-β-carotene (13).

In the products formed during the bleaching of β-carotene by soy-bean lipoxidase in the presence of linoleate, Friend (14) found several epoxides which were probably *cis-trans* isomers of aurochrome the di-furanoid epoxide of β-carotene and three major pigments resembling spectroscopically β-apocarotenals. These may be unsaturated ketones rather than unsaturated aldehydes, possibly with the retro structure.

Six geometrical isomers of vitamin A and retinene are now known: the all-*trans*; two unhindered mono-*cis* isomers, neo-a (13-*cis*) and iso-a (9-*cis*);

[1] The survey of the literature pertaining to this review was completed on November 1st, 1956.

[2] In dealing with *cis-trans* isomerism, most workers adopt Karrer's system of numbering, corresponding to that officially adopted for the carotenoids [*Chem. Eng. News*, **24**, 1235 (1946); see also (1)]. Wald numbered the double bonds of the side chain, Δ1 to Δ5, commencing with that in the β-ionone ring [Wald, G. W., *Ann. Rev. Biochem.*, **22**, 497 (1953)], while Quaife [Quaife, M. L., *Ann. Rev. Biochem.*, **23**, 215 (1954)] gave the number 1 to the terminal alcoholic carbon in the side chain.

[3] The essential fatty acids are not reviewed because of space limitations.

two hindered *cis* isomers, neo-b (11-*cis*) and neo-c (11, 13-di-*cis*); and the unhindered di-*cis* isomer, iso-b (9,13-di-*cis*). The assignment of the 7-*cis* (15) or the 11,13-di-*cis* (16) configuration to the neo-b isomer reported last year (17) has not proved correct, and synthetic studies by Oroshnik (18) have shown it to be the 11-*cis* isomer. This was checked by Wald who condensed the corresponding aldehyde, neoretinene b, with opsin to yield rhodopsin (19, 20). Hubbard agrees with this finding (21). In an application of the Arens & van Dorp synthesis of vitamin A acid (22) using C^{14}, three radioactive isomers labelled in the 14 position are produced, the all *trans*, the 13-*cis* (neo-a vitamin A) and the 9-*cis* (23). Using four isomers of vitamin A, five of retinene, and four of retinene oxime, Hubbard (21) has studied the ease of iodine-catalysed isomerization of the various double bonds. The *cis* configuration of neo-b vitamin A (11-*cis*) is the most labile and iso-a vitamin A (9-*cis*) the least. The retinenes isomerize faster than vitamin A or retinene oximes. *Cis-trans* isomerization of conjugated compounds, including the carotenoids, has been reviewed (24). The rates of reaction of maleic anhydride with vitamin A alcohol and its esters have been studied by Plack (24a). In saponified vitamin A extracts from different oils, differences in reaction rate enable a separation of "fast" and "slow" reacting isomers to be made. The fast reactors have the *trans* configuration at bonds 11 and 13, i.e. the all-*trans* and 9-*cis* isomers. This method permits a partial separation of the isomers more simple than that of fractional chromatography and molecular distillation.

In the routine estimation of carotene and vitamin A emphasis has been on speed, extraction in the presence of surface active agents without preliminary saponification (25), and even visual assessments of chromatograms (26). Useful tables which facilitate the application of the Morton-Stubbs correction for irrelevant absorption in the spectrophotometric determination of vitamin A suitable for both the U.S.P. and B.P. standard methods have been devised by Rogers (27). Surface active agents have been used to facilitate the chromatographic separation of vitamin A alcohol and ester (28). Petracek & Zechmeister (29) have listed the partition coefficients of forty carotenoids and their derivatives between hexane and 95 per cent methanol, useful as a quantitative tool in the identification of the carotenoids. A modification of the AOAC method for the chromatographic determination of carotene in the presence of diphenyl-*p*-phenylenediamine has been reported (30). A method of detecting this antioxidant has also been described (31). The results obtained by the present AOAC chromatographic method for β-carotene are lower than those obtained by older methods based on solvent partition, and the presence of zeaxanthin, neo-β-carotene B and neo-β-carotene U in the epiphase was responsible (32).

Ames & Harris (33) have developed an improved bio-assay for vitamin A, the "slope-ratio liver-storage bio-assay" in which new statistical design has been employed. The liver vitamin A measured with the Carr-Price reagent is linearly related to dose over the range 500 to 10,000 I.U. vitamin A.

Precision was high (±2.9 per cent). Comparative bio-assays of a number of vitamin A preparations including esters and geometric isomers, using the U.S.P. growth method and the new technique, showed no systematic differences (33, 34), but an investigation of provitamins A was not made. In terms of speed, precision, and simplicity the assay of vitamin A in many materials by the liver storage technique is superior to assay by growth response and Ames & Harris contend that the latter will now be replaced. This contention may be questioned since there is certainly not a universal parallelism between the abilities of carotenoids to promote growth and their ability to produce stores of vitamin A (cf. 35). Liver storage of vitamin A was used to test vitamin A preparations and the superiority of emulsions was shown (36).

Occurrence and stability.—Studies by Kon *et al.* (37, 38) on vitamin A in marine invertebrates have been extended to include the phylum Mollusca. Vitamin A was demonstrated chemically for the first time in some members of the classes Loricata and Gastropoda, mainly concentrated in the digestive gland and visceral mass. Analysis of photoreceptor tissue of species in the class Lamellibranchiata showed vitamin A to be absent, suggesting that here it plays no part in vision as it does in the phylum Crustacea and the class Cephalopoda of the Mollusca. Why some molluscs manage quite well in the absence of vitamin A or its precursors while others, even of the same class, require it (37) is intriguing. Examination of the carotenoids of Brazilian species of Gastropoda by Villela (39) showed carnivorous species to contain xanthophyll and its esters, and herbivorous ones carotene. Fisher *et al.* (38) showed that members of the Cephalopoda contained vitamin A as the ester in the liver, as the alcohol in the eggs, and as the aldehyde (retinene) in the eyes. The cephalopod vitamin A is probably all-*trans* (38). In the marine crustaceans the presence of *cis* isomers of vitamin A has been confirmed by biological assay and their further differentiation achieved (40). The *cis* isomers, which react slowly with maleic anhydride, of species of marine crustaceans had biological potencies ranging from 25 to 44 per cent of that of all-*trans* vitamin A. The studies of Massonet (41, 42) have shown that the biological activity of the oil from the hepato-pancreas of the crustacean *Aristeus* was extremely low and the antixeropthalmic activity of extracts appeared comparable to that of astaxanthin esters. Using the small carnivorous fish *Gambusia* as an assay animal and the increase in the vitamin A content of its eyes as an assay technique, Granguad & Massonet (43) found that whole *Aristeus*, or astaxanthin prepared from it, had biological activity, and suggest that vitamin A_2 in the fish is formed from the enolic form of astaxanthin.

An unusual form of vitamin A activity in herring eggs was discovered (44). Potency, determined chemically, was about one fifth that determined biologically, but the fat fraction of the eggs had no activity whatever. The vitamin A stored in the liver of rats and chicks when whole eggs were given was indistinguishable from that stored when vitamin A acetate was supplied,

and the vitamin A-active substance was water- and saline-soluble. Treatment of the eggs with proteolytic enzymes did not increase the Carr-Price reading. The nature of the "hidden vitamin A activity" is not known. A water-soluble factor which increases the liver storage of vitamin A in the chicken has also been found in "fish solubles." This differs from the above finding in that the biological activity of the "fish solubles" is lower than the chemically estimated activity (45).

The amounts of vitamins A_1, A_2 and neo-A in shark liver oils were determined (46) and the distribution of vitamin A_2 in a variety of Indian freshwater fishes studied (47). Most of the vitamin A_2 in the liver was present as the ester and vitamin A_1, when present, was predominantly as the alcohol. Predatory fish contained the most vitamin A_2. The photosensitive pigment of the Australian gecko, *Phyllurus milii*, has an absorption maximum at 524 mμ and the product of bleaching appears to be retinene 1 (48). Studies have been made of the vitamin A content of cow milk in Israel (49), New Zealand (50), and India (51) and each emphasizes the importance of the carotene supply in determining the amount secreted.

A problem comparable with the "lard factor problem" [c.f. Boyer, (52)[arose when vitamin A could not be detected chemically or physically in beef fat and mutton fat although the fats possessed biological activity. Concentration of the vitamin A from the nonsaponifiable fraction of the fat using reversed phase partition chromatography showed that biological activity did, in fact, result from the presence of the vitamin (53). Krause (54) has reported that radioactive xanthophylls from alfalfa when fed to rats give rise to a small radioactivity of the liver vitamin A. Liver vitamin A was also labelled when a radioactive fraction of the nonsaponifiable matter of alfalfa, free of β-carotene and xanthophylls was given. The liver vitamin A was labelled to a small but significant extent when acetic acid labelled with C^{14} was given (55). The method used to isolate vitamin A in these experiments was that of Powell *et al.* (56) which gives a product of 95 per cent purity. Contamination of the vitamin A with cholesterol seems a likely possibility, as the latter would certainly be labelled with radioactive acetate.

The stability of vitamin A in fish liver oils is adversely affected by traces of metals in the ascending order Zn, Sn, Al, Cu, Ni, and Fe (57). Stability of carotene in alfalfa meal was improved by the addition of animal fats, whereas vegetable fats gave no protection (58). Coating of poultry feeds with fat-soluble materials protected the vitamin A provided the fatty materials had not high melting points (59). Variations in losses of carotene in a stack of alfalfa meal during storage were related to temperature and moisture gradients (60).

Absorption and physiological aspects.—Bieri & Pollard reported (61), that carotene when given intravenously as an aqueous dispersion could be converted into vitamin A by rats and rabbits at a site other than the intestine. Species differ in their ability to make the conversion but the mechanism of conversion appears similar after oral dosage or intravenous injection

(62). Hepatectomized-eviscerated rats and rats in which all the major blood vessels to the lower half of the body were ligated could still convert carotene to vitamin A (63). McGillivray *et al.* (62) have shown that the site of conversion is not the thyroid gland, and some other organ or tissue in the anterior half of the body must be involved. The lung had already been suggested as a site for the conversion (64). In the baby chick, the yolk sac acts as a store of vitamin A and its removal increases the sensitivity of the chicks to vitamin A deficiency (65). Injections of carotene in baby chicks leads to increases in liver vitamin A and it is suggested that the yolk sac might be a site of the conversion (66). This indicates that, contrary to the report of Mann (67), the chick can convert dietary carotene to vitamin A on the first day after hatching.

A number of factors which might affect the conversion of dietary carotene to vitamin A has been studied. The technique in most of these studies has been to measure liver and kidney storage of vitamin A when constant carotene is supplied. This means that the over-all absorption, conversion and deposition is measured. In this way vitamin B_{12} has been found to have a small effect (68, 69). Increasing the protein content of the diet from 11 per cent to 22 per cent increased storage fourfold, and restriction of the intake of a high protein diet increased retention twofold (70). Booth (71) has shown that high food intakes and faster passage of food through the gut decrease the "apparent absorption" of carotene, measured as the difference between the amount ingested and that excreted. Ascorbic acid had little effect, but addition of a synthetic vitamin B-complex mixture produced an increased retention (72). Small amounts of diphenyl-*p*-phenylenediamine and, to a much lesser extent, 2,6-di-*tert*-butyl-4-methyl-phenol also increased liver storage of vitamin A when carotene was supplied (69). High (73) showed, however, that fat-soluble antioxidants reduced the liver storage of vitamin A to by 50 to 60 per cent when carotene was given, whereas water-soluble antioxidants had no effect. The liver storage of dietary vitamin A was unaffected and it was concluded that the effect of the fat-soluble antioxidants was due to interference in the conversion of carotene to vitamin A. Calves given methionine or choline stored more vitamin A from dietary carotene than controls, but lecithin, choline, or methionine had no effect on storage of vitamin A when carotene was not given and vitamin A was supplied in the diet (74). Chlortetracycline (aureomycin) increases the biological effectiveness of vitamin A by a mechanism other than that of increased absorption from the gut (74). Increased conversion of dietary carotene to vitamin A in the intestine was found by Chanda to be responsible for the increase in blood vitamin A which occurs when chickens are given L-thyroxine (75). The foregoing results suggest that dietary and endocrine factors can affect the intestinal conversion of carotene to vitamin A, but the techniques have not always excluded other explanations.

The transport of vitamin A in blood and lymph and its deposition in the tissues have been studied. High *et al.* (76), using the technique of McGugan &

Laughland (77), have investigated the hydrolysis and esterification of vitamin A by tissue homogenates *in vitro* and suggest that the natural ester is converted to shorter chain length esters in the liver before hydrolysis to the alcohol. Some hydrolysis of the acetate ester was noted in the blood *in vitro*. During vitamin A depletion, the vitamin A ester of the liver disappeared rapidly whereas the vitamin A alcohol of the blood plasma remained fairly constant. It is thought that specialized carrier proteins are responsible for the maintenance of concentration of vitamin A alcohol in the plasma (78), as suggested by Ganguly *et al.*, several years ago. Studies of the distribution of vitamin A and carotenoids in human plasma showed that whereas 90 per cent of the carotenoids (β-carotene, lycopene, lutein and traces of α-carotene) were associated with the α- and β-lipoprotein fractions, only 10 to 15 per cent of the plasma vitamin A could be found in the α-lipoprotein fraction and negligible quantities in the β-lipoprotein fraction. Vitamin A of human plasma is thus associated with a protein or proteins other than the α- and β-lipoproteins (79). Blocking the reticulo-endothelial system with India ink interferes with the storage of vitamin A ester in the liver but does not affect storage of vitamin A alcohol, which lends further weight to the hypothesis that the ester and alcohol of vitamin A are associated with different liver protein fractions (80). A separation of the bound forms of the ester and alcohol in liver has been achieved (80). The distribution of vitamin A alcohol and esters within the liver cell of human subjects has been studied by Krause & Sanders (81). Higher proportions of the free alcohol were found in normal than in diseased liver but the proportions in the fractions did not change. The cytoplasmic fraction contained the most vitamin A, in contradistinction to the distribution of vitamins E and K (*vide infra*).

Deficiency and metabolism.—Estimates of the requirements of rats for vitamin A depend on the criteria used to assess the deficiency state, and such estimates range from 10 to 1000 I.U./kg. body weight (82). Irving & Richards (83) have studied the effects of relative and absolute deficiency of vitamin A on incisor teeth, maxillary fundic alveolar bone, the medulla oblongata, and the nasolacrimal duct, and have shown that not only do hard tissues, nervous tissue, and epithelial tissue require different amounts of vitamin A to maintain a normal histological appearance, but the relative sensitivity of the tissues changes as the animal grows. They make the generalization that "the requirement [of vitamin A_1] is a function of that of the most sensitive tissue at the time of the experiment." Light microscopy and electron microscopy have been used to study the morphology of corneal epithelium in vitamin A deficiency (84, 85) and morphological abnormalities have been noted in the mitochondria in the intermediary keratinizing layer. Fluorescence microscopy has shown the presence of vitamin A in endothelial cells, and vitamin A can reverse the effect of citral which causes damage to the vascular endothelium, in both rabbits and monkeys (86). The effect of citral appears to depend on the terminal C:CH·CHO group, and its toxicity is also reversed by sulphydryl compounds. Leach & Lloyd

(86) suggest that citral may act as a retinene competitor. Replacement therapy with oestrogens in ovariectomized rats has shown that the metaplastic change in the uterus in intact rats deficient in vitamin A is a synergistic effect in which both vitamin A deficiency and oestrogen participate (87). Electroretinography has been used as a diagnostic method in India, and it was concluded that xerosis in the human subject is not a direct manifestation of vitamin A deficiency (88).

Wald & Brown (89) have elegantly demonstrated the formation of rhodopsin from neo-b-retinene (11-*cis*) and opsin, the bleaching of rhodopsin (which appears to be a first-order reaction) to form all-*trans* and smaller amounts of other isomers of retinene and opsin, and the reduction of the retinene to vitamin A. High salt concentrations have been shown by Bridges (90) to modify the bleaching of rhodopsin to give a stable orange photoproduct with a maximum at 482 mμ. This is not indicator yellow, to which the orange intermediate can be transformed, but conforms to *meta*-rhodopsin (λ max. 480 mμ) which is one component of the mixture of *lumi*-rhodopsin, *meta*-rhodopsin, and *iso*-rhodopsin, called transient orange. Hagins (91) has measured photochemical events in the retina following light flashes. A substance resembling *meta*-rhodopsin was produced by flash photolysis which faded exponentially with a half life, at 12°C., of 20 msec. Calculations indicate that at 37°C. the half life would be 120 μsec. The photochemical changes are thus completed long before any electrical response of the retina can be detected. Flash photolysis has also been used with rhodopsin and retinene in solution to study the transient photochemical changes, and the results can be interpreted in a similar way (92).

Hypervitaminosis.—Administration of excess vitamin A produces skeletal fractures, and metabolic methods have now been used to study the mechanism. Large losses of body calcium, phosphorus, and nitrogen occurred in hypervitaminosis in the rat; the serum calcium and phosphorus were undisturbed but the activity of the bone alkaline phosphatase was increased (93). No marked changes occurred in the chemical composition of the bones, which decreased in thickness. Fell has reviewed the effects of excess vitamin A in organized tissues cultivated *in vitro* including its effect on bone (94). The effect of hypervitaminosis-A in rats was accentuated by diphenyl-*p*-diphenylenediamine in the diet but not when α-tocopheryl acetate was supplied (95).

The lameness of lambs on pasture in New Zealand was first ascribed to a quantitative antirachitic effect of β-carotene (96, 97). The rachitogenic effect of carotene has not been confirmed (98, 99, 100). Hypervitaminosis A is a possible cause of the lameness since the intake of vitamin A activity under these conditions is 1 to 2×10^6 I.U. daily. In hypervitaminosis A in the human subject, bone changes and joint pain occur though these signs may be less evident than changes in the skin and mucocutaneous junctions (101, 102).

Deficiency states in farm animals.—Visible signs of vitamin A deficiency have been found under natural conditions in sheep (103) and calves (104).

Damage to the peripheral nervous system and optic stenosis in cattle have also been recorded from the field (105) but urethral occlusion in cattle is apparently not a result of vitamin A deficiency (106). In bovine hyperkeratosis induced by chlorinated naphthalenes (107) the collagen content of the skin is subnormal and the plasma vitamin A and ascorbic acid are reduced, suggesting that these agents interfere in the conversion of carotene to vitamin A, the synthesis of vitamin C, and the formation of collagen (108). Low serum carotenoids and vitamin A concentrations are found to be associated with abortion in mares (109) and the placental transmission of vitamin A and carotene in the horse has been studied (110). The supply of vitamin A to the suckling pig and its relation to the plasma concentration of vitamin A in the sow have also been studied (111, 112).

Vitamin D

The antirachitic compound formed by the action of floridin (activated clay) on cholesterol (113, 114) has been shown by Raoul *et al.* (115) to be an α,β-ethylenic ketone linked to a tertiary alcohol (9:10-*seco*-cholest-5-en-7-one-3β,10-diol). This ketone has been isolated from crude liver oil of red tunny in larger amounts than vitamin D_3 (116). Its biological activity, assayed both with rats and chicks, was one tenth that of vitamin D_3. The ketone is very easily converted in alkaline solution to an enolic form which in turn yields a calcium salt (117). This enolic form has a biological activity in the rat and chicken of 40,000 I.U./mg. compared with 4000 I.U./mg. for the non-enolized ketone. This means that the enolic form has an antirachitic value greater than that of vitamin D_3. Implications of this finding on the interpretation of vitamin D analyses is clearly important (116) and Raoul and his co-workers (117) suggest that the enolic form plays a considerable part in the transport of calcium from an aqueous to a lipide medium, as in the intestinal lumen.

Louw *et al.* (118) have described the conversion of some new Δ^5 steroids, with aliphatic side chains ending in a tertiary amine, into provitamin D. Antirachitic activity after ultraviolet irradiation was reduced when a methylene group or groups was introduced into the side chain or when a dimethylamide group was reduced to a dimethylamine. Antirachitic activity of vitamin D_2 was destroyed by progressive sulphonation (119).

A simple chemical method for separating vitamin D_2 from vitamin D_3 in binary mixtures has been described (120), as well as a colour reaction of vitamins D_2 and D_3 with iodine-ethylene dichloride (121). Ergosterol, 7-dehydrocholesterol, stigmasterol, and sitosterol did not interfere, but the sensitivity of the method was lower than with the antimony trichloride reagent.

Metabolism.—Kodicek (122) has continued his studies with uniformly labelled ergo-calciferol-C^{14} (123), estimating the vitamin by paper chromatography (124). Only 30 per cent of an oral dose of ergo-calciferol was recovered as vitamin D after 24 hr., though the C^{14} was accounted for quan-

titatively, mainly as "breakdown products," mostly in the faeces (122). $C^{14}O_2$ was not respired (125). Differences in the properties of the breakdown products (solvent distribution, digitonin precipitation) indicated that the metabolism of the vitamin in the liver differed from that in the intestinal wall. The bulk of the unchanged vitamin was in the liver (5.7 per cent of the dose) but in those tissues intimately concered with phosphate turnover—bones, intestine, and kidney—there were significant amounts (124). The distribution did not parallel the lipide content of the organs (125). Biological and chemical methods were also used by Cruickshank & Kodicek (126) to study the distribution of vitamins D_2 and D_3 following oral and intramuscular administration. Twenty-four hours after injection, the vitamin appeared in intestinal tissue, which suggests that its action on the mineral metabolism of intestinal cells is local. No differences in tissue distribution were noted whether vitamin D_2 or vitamin D_3 was the source of activity or whether the vitamin was given in oil or aqueous dispersion.

Sterol mixtures isolated from the preputial glands of mice and anal glands of guinea pigs contained 7-dehydrocholesterol in very large amounts as well as cholesterol and cholest-7-en-ol (127). The protected position of these glands would seem to preclude their exposure to ultraviolet rays, but their richness in provitamin D suggests a function in meeting vitamin D requirements. The absorption of topically applied vitamin D is reported to be extremely efficient and rapid (128).

Deficiency.—The proceedings of a symposium on bone structure and metabolism (129) have been published, which deals also with diseases of bone in man and animals.

Nicholaysen *et al.* (130, 131, 132) have studied calcium metabolism in the rat. With diets containing adequate calcium and phosphorus, vitamin D increased calcium absorption and resulted in heavier rats with more fully mineralized bones (130). A depletion of body calcium caused by reduction of the calcium content of the diet could not subsequently be restored by an increase in the calcium content of the diet without vitamin D (131). Nicolaysen concluded that calcium absorption and retention is regulated not only by vitamin D but by an "endogenous factor" and that the rate of calcium absorption is not simply determined by the progressive calcification of the skeleton (133). Nicolaysen (132) has also shown that calcium retention is unaffected by old age in rats, except when food intake is grossly reduced or the animals are close to death. This is contrary to findings of Liu & McCay (134) in old dogs, which had difficulty in maintaining calcium equilibrium, and of Henry & Kon in old rats (135). Possibly Nicolaysen's results reflect an adaptation of calcium metabolism in which the previous pattern of skeletal accumulation of calcium must be considered. In the cat, evidence suggests that vitamin D requirements decrease with age (136).

Steenbock & Herting (137) have studied the effect of vitamin D on rats given diets containing less than 0.02 per cent of calcium. Despite this severe deficiency of calcium, the addition of vitamin D increased growth, more so

in fact than the addition of calcium to the diet or alterations in the phosphorus supply. These results, in accord with those of Bellin *et al.* (138), suggest that vitamin D has an effect on metabolism other than on calcification mechanisms, and Steenbock & Herting think that an effect of vitamin D in citrate metabolism may be involved. The antirachitic effect of citrate on cereal diets may be explained by its effect on intestinal phytase (139) but an effect is still present when the dietary phosphorus is in inorganic form. Furthermore, citrate can reduce calcium absorption when low phosphorus diets are given, and under these conditions citrate is rachitogenic (140).

Studies by de Luca *et al.* (141) on citrate oxidation in kidney tissue of rachitic and normal rats showed that vitamin D reduced the oxidation of citrate but not of oxaloacetate. The exchange of citrate in bone *in vitro* has been studied by Armstrong & Singer using C^{14}-labelled citrate (142). A competition between phosphate and citrate on the mineral crystal surfaces of bone was demonstrated. Aconitate also competed but not succinate or glutarate. It is concluded that the bone citrate is located at the crystal surfaces, that it is in dynamic equilibrium with body fluid citrate, and that the source of bone citrate is in extra-osseous tissue.

Experiments with rats on a low phosphorus rachitogenic diet showed that cortisone had a marked rachitogenic effect (143) whereas, when rickets was produced by low calcium diets, very little effect was seen (144). Growth hormone administered to rats on a low calcium diet free of vitamin D resulted in increased growth of soft tissues and size of bones but there was no change in calcium retention (145).

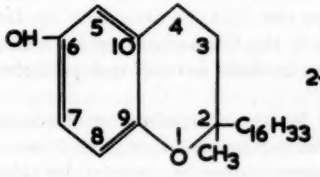
Hypervitaminosis.—The nitrogen, calcium, and phosphorus metabolism of rats given large amounts of vitamin D_2 was studied by Chitre & Damle (146) who showed that only the exogenous protein metabolism was affected and that on protein-free diets, no increase in urinary nitrogen excretion occurred. In dogs given excess vitamin D_2, glomerular filtration and renal flow were reduced (147). At a later stage, maximal tubular reabsorption of phosphate failed. These findings are presumably secondary to the renal calcification which occurs in the hypervitaminosis. The distribution of calcification in the vascular system of rats given excess vitamin D_2 showed some vessels to be more resistant than others (148).

Idiopathic hypercalcaemia in infants has been described in detail including its pathology, clinical biochemical findings, possibly subdivision into types, and the effects of treatment with low calcium diets, ethylenediaminetetracetic acid or with cortisone (149 to 153). The disease has many aspects comparable with those in hypervitaminosis-D, and some evidence of overmedication has been found. In other cases, hypersensitivity to vitamin D is predicated. In dogs there is a wide gradation of response to a toxic dose of vitamin D (147) so that differences in sensitivity are by no means unlikely. The overfortification of British proprietory foods with vitamin D, and the supply of cod-liver oil free of cost under the National Health Scheme, ensures that many infants must receive at least eight times the recommended allowance of 500 I.U. vitamin D daily (154). The high incidence of hy-

pervitaminosis-D, recorded in Britain and Europe, has not evidently been met in the United States. Nevertheless, the evidence incriminating vitamin D is, at the best, circumstantial. Thus, Forfar *et al.* (155) have pointed out differences between true hypervitaminosis-D and the idiopathic disturbance in the infants and have suggested that the primary disturbance is in cholesterol metabolism, while Fyfe (156) has reported abnormalities in vitamin A metabolism in the disease. Abnormalities of both cholesterol and vitamin A metabolism could be secondary to the renal damage, which invariably is present.

Vitamin E

Analysis.—The seven known tocopherols are listed in Figure 1 the latest to be found in natural products being 7-methyl tocol (157). The prefix ϵ is now reserved for the 5-methyl tocol, and its earlier use to denote the synthetic nonmethylated tocol (158) has been abandoned. An improved process for synthesis of α-tocopherol by condensation of phytyl chloride with trimethyl hydroquinone in the presence of BF_3 catalyst has been described (159).



I

2-methyl-2-[trimethyl-tridecyl]-6-hydroxychromane (tocol)

(alpha)	α-tocopherol	5+7-8-trimethyl tocol
(beta)	β-tocopherol	5-8-dimethyl tocol
(zeta)	ζ-tocopherol	5+7-dimethyl tocol
(gamma)	γ-tocopherol	7+8-dimethyl tocol
(epsilon)	ϵ-tocopherol	5-methyl tocol
(eta)	η-tocopherol	7-methyl tocol
(delta)	δ-tocopherol	8-methyl tocol

Fig. 1. The known tocopherols.

Analytical methods for the tocopherols have been studied and the limitations of older methods indicated. The two-dimensional absorption-partition chromatographic method (160), separates the tocopherols, first by absorption according to the number of methyl groups *ortho* to the hydroxyl group, and secondly by partition into isomeric groups without reference to the position of the methyl groups (161). This separates the tocopherols into five groups, in which β- and γ-tocopherols can be separated after coupling with diazotised-*o*-dianisidine by 2-dimensional chromatography of the products (161). It is possible that η-tocopherol could be separated in a similar way. Eggitt & Norris (162) have used reversed phase partition chromatography in which a liquid paraffin-aqueous ethanol system is supported on kiesel-

guhr made hydrophobic with dimethyl-dichlorosilane to isolate the three isomeric groups. The dimethyl tocols are then quantitatively resolved in two steps: first, by coupling with diazotised-*o*-dianisidine, the γ-tocopherol is separated, and secondly, by formation of the nitroso compounds, the β- and γ-tocopherols are obtained [ζ-tocopherol does not form a yellow nitroso derivative (160)]. ζ-Tocopherol is then determined by difference. In a comparable scheme for the separation of monomethyl tocols, resolution of ζ-tocopherol would be difficult since like δ-tocopherol, having a free non-methylated 5 position, it couples with diazonium salts and all three monomethyltocols form yellow nitroso derivatives. Nevertheless, Eggitt & Norris' (163) system makes possible the separation, with a single chromatogram, of individual tocopherols except perhaps η- and δ-. The authors have used the method to isolate ε-tocopherol in 5 mg. batches.

The oxidation of α-, β-, γ-, δ- and ε-tocopherols in the Emmerie-Engel test has been studied (162) since this is of such importance in their determination. When C-5 is methylated (α-, β- and ε-) tocopheroxides are formed and converted cleanly to the quinones. With C-5 vacant more extraneous reaction products are formed, so that with δ-tocopherol these become the major products. A collaborative test (164) to determine the factors for the various tocopherols to be used in the Emmerie-Engel determination shows that γ-tocopherol and more particularly δ-tocopherol produce much more colour for the above reasons.

The Furter-Meyer method for the determination of tocopherols (165) involves their oxidation to the *o*-quinones. Eggitt & Norris (162) have shown that total tocopherol figures cannot be obtained by this method because of the wide variation in the reactions of individual tocopherols, particularly of δ-tocopherol.

A method in which the non-saponifiable material of serum is chromatographed on paper impregnated with ferric undecylate in spermaceti has been described (166). In this way, vitamin A alcohol, carotenoids, and steroids are separated from α-tocopherol. A modification of the Emmerie-Engel procedure using ferricyanide has been published (167). Specificity is claimed (168) for a rapid clinical method for determination of tocopherol, which involves spectrophotometric measurement of the colour developed on adding phosphomolybdic acid in glacial acetic acid to the non-saponifiable material of serum. A method for the phosphate ester of α-tocopherol has also been described (169) which permits its separation from adenosine and glucose esters in tissues. Methods for the analysis of pharmaceutical preparations of tocopherol have been reviewed by Bolliger & Bolliger-Quaife (170). Classical analytical methods have also been reviewed (171).

While rapid methods find considerable clinical use, exact quantitative analysis of the tocopherols present in natural products is essential. This involves separation of the different tocopherols and is complicated by oxidation products. The so-called "origin spot," found in the chromatograms of Brown (172) and Eggitt & Ward (173), though it reacts with the Emmerie-Engel reagent, is not a tocopherol (161). The α-tocopherol quinone and hy-

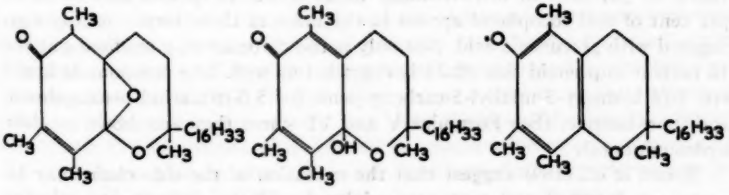
droquinone can be separated chromatographically [Harrison *et al.* (174)], but in the system employed the epoxide was not sufficiently stable to give a single spot. The quinone and epoxide are more usually determined following their reduction to tocopherol, as in analyses by Bouman & Slater (175). Determinations of the total tocopherol content of natural products, using the Emmerie-Engel or the Furter-Meyer methods of analysis, may have doubtful biological significance, and extensive characterization of the tocopherol "patterns" of natural products is necessary.

From the limited analyses by these methods it appears that plant products can be divided into groups according to their tocopherol pattern (161). One contains those with the α-, γ- and δ-pattern (5,7,8-, 7,8- and 8-methyl), and a second those with the α-, β-, ζ- and ϵ-pattern (5,7,8-, 5,8-, 5,7- and 5-methyl). Rice has the pattern α-, ζ-, η- (5,7,8-, 5,7- and 7-methyl) (157) and may be a member of another group. Methylation of the three monomethyltocols (176) gives rise to similar patterns except that the 5,8-dimethyl tocol (β-tocopherol) is not formed on methylation of ϵ-tocopherol. Their biosynthesis may proceed by a stepwise methylation of the chroman nucleus but in any event biogenesis of the tocopherols is a new field opened by these methods.

Holman, in a compilation of data on the vitamin E content of human foods, carefully distinguishes between chemical determinations of tocopherols and biological assays (177). Biological assay and chemical estimation of tocopherol in alfalfa gave similar results (178), which is understandable since 95 per cent of the tocopherol of alfalfa is α-tocopherol (172).

No further information is available (179) regarding the biological activity of the newer tocopherols (ζ-, ϵ- and η-). Using the effect of intraportal injection of tocopherols on reversal of the respiratory lesion in dietary liver necrosis as an assay method, α-, β-, γ- and δ-tocopherols gave biological activities comparable with those obtained by more classical methods (180). An assay method based on testis weight in vitamin E-deficient rats was used to assess the vitamin E content of flour (181).

The oxidation products of the tocopherols.—The possibility that α-tocopherol has a biochemical function as a component of a reversible oxidation-reduction system has always stimulated interest in its oxidation products.



II α-Tocopheroxide (the epoxide ring may be on carbons 8 & 9)

III hemiketal intermediate

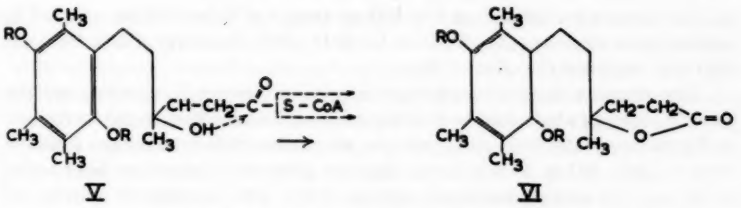
IV chromanoxyl free radical

Chemical studies by Boyer and his associates (174) have shown that stable electromotive potentials are not obtained in α-tocopherol-α-tocopheroxide mixtures. α-Tocopheroxide (II) readily forms the α-tocopheryl quinone (ρ-quinone) in weakly acid solutions; but under similar conditions, when ascorbic acid is present, α-tocopherol is formed. A common intermediate with a hemiketal structure (III) is suggested. A reversible system, linked to ascorbate or other reducing agent, might conceivably occur *in vivo*. It is in some ways comparable to earlier systems that have been suggested (182, 183). On the basis of oxidation of α-tocopherol by benzoyl peroxide Inglett & Mattill (184) suggest that a free chromanoxy radical (IV) is formed as intermediate. Besides the tocopheroxide, the ρ-quinone, hydroquinone, and *o*-quinone (tocopherol-red or toco-red) several other products of oxidation of tocopherol are known but not characterized. They include one isomer not identical with α-tocopheroxide (184), a non-reducing compound (184), and an inert non-reducing product with an absorption spectrum resembling that of α-tocopherol (174). Nor has the chemical constitution of all five products of the oxidation described by Frampton, Skinner & Bailey (185) or the dimer earlier described by Boyer (183), and since noted by Eggitt & Norris (162), yet been elucidated.

Oxidation of the tocopherols *in vivo* has been studied by labelling the 5-methyl group of *d*-α-tocopherol with C^{14}. The fact that this label remains intact during the metabolism of the tocopherol (186) is of interest since the C-5 position of the chroman nucleus is usually regarded as particularly vulnerable even when substituted, as instanced by the following examples. The red quinone found in auto-oxidising cottonseed oil is the 5:6-orthoquinone of δ-tocopherol (184). Like α- & δ-tocopherols, ε-tocopherol forms red orthoquinones with nitric acid in ethanol. Both β- and ε-tocopherols produce a brilliant violet unstable colour (not the quinone) with nitric acid in chloroform (162). In these two, C-5 is substituted to block *o*-quinone formation and C-7 is unsubstituted. Certainly the *o*-quinone (toco-red) is not formed on metabolism of α-tocopherol in the body (187).

Studies by Milhorat & his colleagues (188) with labelled tocopherol in rabbits have shown that appreciable quantities of two oxidation products occur in the urine and these have been found also in man (186, 189). Virtually 99 per cent of intravenously injected *d*-α-tocopherol and 20 to 30 per cent of oral tocopherol appear in the urine in these forms, mostly conjugated with glucuronic acid. Not only is the chroman ring oxidised but the 16 carbon isoprenoid side chain is degraded as well. The compounds found are 2-(3-hydroxy-3-methyl-5-carboxy-pentyl)-3,5,6-trimethyl-benzoquinone and its γ-lactone. (See Formulae V and VI where they are shown as their hydroquinones).

Simon *et al.* (189) suggest that the oxidation of the side chain may be coenzyme A-linked and may proceed by β-oxidation following a primary oxidation of one of the terminal methyl groups of the side chain to a carboxyl group. Termination of the β-oxidation two carbons short of comple-



V VI

R may be H or a glucuronic acid residue

tion is thought to occur as the result of formation of the stable lactone. The acid probably arises by hydrolysis of the lactone, and it is suggested that the compounds are excreted as the hydroquinones conjugated with glucuronic acid, which would be oxidised easily to the quinones after hydrolysis.

Metabolism.—The visible and histopathological signs of many vitamin E deficiency syndromes are modified by dietary factors other than tocopherols. It is reasonable to suppose that such interactions also occur at the molecular level, and that apparent contradictions in the literature reflect on the validity of the assumption that metabolic defects are due solely to lack of dietary tocopherol.

Some progress has been made in separating the effects of unsaturated fats from those of vitamin E. Irving & Budtz-Olsen (190) have shown that the effect of cod-liver oil on tooth pigmentation and degeneration of the enamel organ in the rat can be reversed by α-tocopherol, but the effect of hake-liver oil cannot, despite the fact that tocopherol reverses the dialuric acid haemolysis test in these rats. Some constituent of the hake-liver oil (which has an unsaturation comparable with that of cod-liver oil) must be involved. Remarkable differences in the effects of vitamin E deficiency were noted by Moore *et al.* (191) when the lard (normally 10 per cent) in the diet was omitted, increased, or replaced by cod-liver oil. Erythrocyte haemolysis by dialuric acid, slight discoloration of the uterus, and testicular degeneration occurred even when signs of an essential fatty acid deficiency were present. Omission of dietary fat protected against dental depigmentation and *post-mortem* autolysis of the kidney (192). Cod-liver oil was the only fat to cause brown discoloration of adipose tissue, but it tended to prevent testes degeneration and uterine discoloration. It was also the dietary component responsible for cardiorespiratory failure of pregnant rats—"rat eclampsia." Crude linoleic acid was equally effective as a causative agent and α-tocopherol prevented the disease (193). Vitamin E improves reproductive performance on fat-free diets and on diets which cause signs of essential fatty acid deficiency (194, 195). The addition of a vitamin B_{12} preparation or an increase in the protein content of the diet had no effect on reproductive performance on a diet free of vitamin E (196).

The substitution of ferrous sulphate for ferric phosphate in the salt mixture of a mouse diet containing adequate vitamin E, changed the incidence

of adult mouse paralysis from 0 to 100 per cent, and this could be reversed by adding trace elements (Zn, Mn, Cu, Co & I) (197). Removal of lard from the diet also reversed the effect (198).

The complex dietary interactions involving vitamin E, cystine, and the protein supply (which lead to liver necrosis on *Torula* yeast diets) have been well confirmed (199, 200) and occur also when rats are fed the fungus *Psalliota lispora* (201). When *Torula* yeast diets are given to chickens no liver lesion develops, but encephalomalacia occurs (178). The necrogenic activity of different yeasts is parallelled by their biological value in terms of their ability to promote liver protein regeneration (199).

The foregoing indicates the difficulties attendant upon the interpretation of overt signs of vitamin E deficiency. This applies also to the interpretation of metabolic data. Thus in a study of oxidative phosphorylation in rabbits on the tocopherol-deficient diet of Mason & Harris, P/O ratios, obtained on oxidation of α-ketoglutarate by liver mitochondria, were significantly depressed in the absence of tocopherol but the site of uncoupling could not be localised. However, fatty fibrotic livers developed in both vitamin E-deficient and more particularly in vitamin E-supplemented animals; it appeared that the basal diet was not only inadequate in some respect other than tocopherol content, but that tocopherol aggravated the effect (203).

With animals on diets consisting of vitamin E-free *Torula* yeast, the failure of the liver (which undergoes necrotic degeneration) to maintain oxygen consumption (204) has been studied. No increase in oxygen consumption followed exposure to various concentrations of 2,4-dinitrophenol, regardless of the initial glycogen content of the slices. It was concluded that the "respiratory lesion" may involve a defect in adenosine triphosphate synthesis (205). Oxidation of tricarboxylic acid cycle intermediates by washed liver homogenates from vitamin E-deficient animals was increased by adenosine triphosphate, while additions of pyrophosphate caused a depression. There was no change in pyrophosphatase activity in the deficiency (206).

Further study by Nason *et al.* (207) of the enzyme system which possesses reduced diphosphopyridine nucleotide cytochrome-*c* reductase activity and which is inhibited by *iso*-octane extraction and is specifically restored by the addition of tocopherol, has shown that large amounts of crystalline bovine serum albumin can also restore activity. Albumin extracted with *iso*-octane was inactive but the lipid extract had activity, albeit somewhat reduced. Tocopherol, together with the *iso*-octane-extracted albumin, or in ethanol solution, restored activity. Crystalline egg albumin and human serum γ-globulin were inactive. The effect of bovine serum albumin is clearly not one of inactivation through non-specific heavy metal chelation of the -SH groups of the enzyme, but is the result rather of a lipid cofactor; indeed, ethylene-diamine-tetracetic acid and calcium phosphate gel had no activity. Bouman & Slater have detected (175) small amounts of tocopherol in crystalline bovine serum albumin obtained from the same source as that used

by Nason & Lehman. The quantity found is not of the same order of magnitude as that necessary for activation of the enzyme, but it is possible, in view of the very considerable surface activity of tocopherols (208) and their ability to form spontaneous monomolecular films, that these small quantities would be effective. Tedeschi & Harris (209) have indicated that mitochondria possess a semipermeable membrane containing lipide. This may well be the site of concentration of the tocopherols in particulate preparations.

Nason & Lehman (210, 211), describing cytochrome-*c* reductase, report that the lipide cofactor from beef heart homogenate has been purified two hundredfold. Ten μg. of this purified cofactor gave 50 per cent restoration of activity whereas 45 μg. of *d*-α-tocopherol was necessary to give the same effect. The lipide cofactor contains no free tocopherol. It is suggested that the site of action of tocopherol and the lipide cofactor is probably between cytochrome-*b* and *c*. The lipide cofactor is present in a wide variety of materials, and diphosphopyridine nucleotide cytochrome -*c* reductases from a number of sources showed tocopherol stimulation. Where tocopherol does substitute for the lipide cofactor it has not yet proved possible to demonstrate its alternate oxidation and reduction but the possibility has not been dismissed. However, it is tentatively suggested by Nason & Lehman that the cofactor or tocopherol acts as cementing material for maintaining cytochromes-*b* and *c* in correct spatial relation for optimal electron transfer. This function is comparable to that ascribed to phospholipides in succinate oxidases (212).

Dinning's isotopic tracer studies of the nucleic acid and protein metabolism of muscle in vitamin E-deficient rabbits have been continued (213). In vitamin E deficiency, formate, phosphorus (214), and guanylic acid incorporation into nucleic acids increased, but glycine incorporation was unchanged. This suggests a greatly increased rate of exchange of the formate of nucleic acids, probably in position 2 of the purine ring. In the experiments with guanylic acid only the guanine moiety was incorporated, not the ribose (215). The specific activity of urinary creatinine was increased in animals deficient in vitamin E (216) a finding in accord with impaired phosphorylation and retention of creatine. Urinary excretion of free amino acids increased before clinical signs of deficiency were apparent (217). The protein of skeletal muscle of vitamin E-deficient rabbits had a higher activity following injection of sodium formate-C^{14} or glycine-1-C^{14}, but the radioactivity of the isolated glycine was unchanged. When formate-C^{14} was injected, an increase in the activity of the muscle methionine and serine occurred in deficient but not in normal rabbits (218, 219). The results are explained in terms of the enhanced excretion of allantoin and creatine, synthesis of both of which involves 1-carbon fragments.

The hydrolysis of glycylglycine and glycylleucine by skeletal muscle extracts and homogenates showed that in vitamin E deficiency, dipeptidase activity was markedly increased (220). The cathepsin activity of muscle is also increased (221). It is suggested that the net breakdown of muscle pro-

tein in the deficiency is due to an equilibrium shift of those enzymes catalysing peptide bond formation and hydrolysis (220).

Erythrocyte haemolysis.—Haemolysis of erythrocytes in vitamin E deficiency has been studied further. In vitamin E-deficient rats, spontaneous haemolysis occurs when their erythrocytes are suspended in saline at room temperature and this is accelerated at higher temperatures. Free oxygen must be present, but hydrogen peroxide or dialuric acid is unnecessary (222). Erythrocytes from vitamin E-deficient chickens are more resistant to spontaneous oxidative hydrolysis; indeed, Muytjens (223) did not observe hydrolysis at all. Dialuric acid haemolysis occurs in the E-deficient chicken (222, 223). Hydrogen peroxide haemolysis of erythrocytes and tocopherol content of the plasma showed inverse correlations in normal full-term and premature infants and in those with steatorrhea, but in unfed premature babies low tocopherol concentrations were not necessarily associated with increased haemolysis (224, 225). This prompted investigation of the catalase content of the erythrocytes in such infants (226) and it was found that where catalase was high, resistance to haemolysis increased. In confirmation of earlier work Rose & György (227) found that a variety of substituted hydroquinones, added to erythrocytes *in vitro*, were effective in preventing hydrolysis (226). An experiment, as yet incomplete, in which adult human subjects were given ~1.9 mg. tocopherol/day (Emmerie-Engel) in natural foods and were compared with a group given a supplement of 15 mg. *d*-α-tocopherol daily, showed that an increase in erythrocyte haemolysis occurred in the unsupplemented group. Again there was a lack of correlation between the tocopherol content of the blood and the extent of haemolysis (228). As an example, supplementation with α-tocopherol of two depleted patients gave a marked fall in haemolysis without significant change in plasma tocopherol. A case of xanthomatous biliary cirrhosis in a woman on a low fat diet for three years (229), who had no tocopherol serum in her and had a positive hydrogen peroxide haemolysis test, suggests that deficiency of tocopherol in adult human subjects may, as in the infant, follow fat malabsorption syndromes. However, the above shows that the various haemolysis tests do not simply assay the tocopherol or antioxidant content of the plasma. In the monkey, vitamin E deficiency (230, 231) leads to anaemia characterized by a fall in the erythrocyte number (with a normal colour ratio) and a marked increase in the neutrophil count. This anaemia is comparable to one due to haemolysis but on giving α-tocopherol phosphate a reticulocyte response occurs, suggesting a more specific role for tocopherol in erythropoiesis (230). A microcytic anaemia with a low reticulocyte count has been noted in vitamin E-deficient chicks (232).

The haemolysis of erythrocytes in vitamin E deficiency is probably related to oxidation of the unsaturated fatty acids of the phospholipid stroma of membrane of the erythrocyte comparable with the haematin-catalysed oxidation of unsaturated fats. In partially purified preparations of the phospholipids of the erythrocyte stroma from vitamin E-deficient subjects,

an increased peroxidation (228) was demonstrated with the 2-thiobarbituric acid reagent (233), and pre-incubation of erythrocytes with ferrous o-phenanthroline increased peroxide haemolysis. The effect of dietary methylene blue in partially preventing haemolysis, *in vitro*, of the erythrocytes of vitamin E-deficient rats (222) is in agreement with the ideas of Tappel (234) on the inhibition of haematin catalysed peroxidation of linoleic acid by phenothiazine derivatives. Methylene blue (a phenothiazine derivative) also protects against haemolysis, *in vivo*, at high oxygen tensions (235). Oxidation of linoleic acid by plant lipoxidase is also inhibited by phenothiazine and α-tocopherol (236). Some phenothiazine drugs inhibit haemolysis of human erythrocytes by lysolecithin *in vitro* (237). Here haemolysis is preceded by a loss of cholinesterase from the surface of the erythrocyte. Since more tocopherol is needed to reverse a positive haemolysis test in the presence of serum, György (238) has suggested that part of the tocopherol in plasma is bound and biologically inactive.

Before the aetiological importance of high oxygen tensions in causing retrolental fibroplasia in premature infants was realised, the condition was ascribed to vitamin E deficiency (239). It now appears that the vitamin E status of the infant must again be considered (240). Taylor showed that rats deficient in vitamin E are more susceptible to the effects of high oxygen tension (235) which, besides causing nervous effects, is associated with haemolysis of the red cells *in vivo*, but not *in vitro*. Haemolysis is prevented by tocopherol but not by methylene blue. As judged by responses to α- and γ-tocopherol, glutathione, and methylene blue, the mechanism of dialuric acid haemolysis differs from the high oxygen tension haemolysis, and it is suggested that SH groups are involved in the latter (241). Pirie & Heyningen (242) have suggested that inactivation of those enzymes, dependent on a thiol group such as lipoic acid, and coenzyme A are involved in retrolental fibroplasia. Furthermore, Olson has shown that the rate of incorporation of S^{35} from labelled cystine into liver coenzyme A is markedly reduced in vitamin E deficiency which may suggest that vitamin E is concerned in the synthesis of coenzyme A (243). Haemolysis induced by excessive doses of vitamin K substitutes is discussed later, but it may be noted that menadione is combined in blood through SH linkages (244). No effect of diet on the incidence of retrolental fibroplasia was found in a group of premature infants with a 36 per cent incidence of the disease (245).

A further defect of vision referable to vitamin E deficiency, as yet only in the turkey, is liquefaction of the lens fibres and degeneration of the lens epithelium (246, 247).

Deficiency states in farm animals.—Muscular dystrophy in farm animals has been reviewed (248, 249). Reviews on the fat soluble vitamins in cattle nutrition (250) and on vitamin E deficiency syndromes (251) in poultry have appeared.

The enzootic muscular dystrophy of suckling calves in the north of Scotland is not associated with abnormally large amounts of unsaturated fatty

acids in the milk fat of the dams but with very low tocopherol contents. No abnormality in the distribution of polyethenoid acids in the blood serum of affected calves was detected (252). In Montana, the tocopherol content of the milk and colostrum of range cattle varied considerably, and the herd with the highest incidence of muscular dystrophy had the lowest tocopherol concentrations (253). The requirement of calves for α-tocopherol, with lard as a source of fat, was placed at 20 mg./day/cwt. body weight (254). This is higher than the requirement noted on milk diets (249, 252), or on diets virtually free of fat (248, 255). Stress factors may contribute to the incidence of dystrophy, and the adrenal cortex may be involved in its aetiology (249). The tocopherol content (Emmerie-Engel) of the milk fat of cows and buffaloes in India varies seasonally, depending on the grass intake, the values being comparable to those obtained elsewhere (256, 257).

In day-old lambs given artificial diets with lard as the source of fat, diphenyl-*p*-phenylenediamine prevented the appearance of muscular dystrophy up to an age of six weeks (258). On natural diets, no correlation was found between the tocopherol content of the blood or milk of the ewes and incidence of the disease, and some ewes with very low blood tocopherol produced normal lambs. Diphenyl-*p*-phenylenediamine given to the ewe had no effect on the tocopherol content of its colostrum or milk, nor was incidence of the disease in lambs affected. Barley in the diet of the ewe was dystrophy-producing (259). Muscular dystrophy has now been recorded in older sheep (260) and under pasture conditions in New Zealand in which the possibility of dietary deficiency of vitamin E seems remote (261). A liver necrosis of sheep of unknown aetiology may be related to dietary deficiency of tocopherol, cystine, and Factor III (262).

An extensive spontaneous myopathy in sheep $2\frac{1}{2}$ to $3\frac{1}{2}$ years old in England (263) may be the only pathological disorder of sheep affected by the disease "scrapies," which hitherto had been regarded as a neurotropic virus infection (264) capable of transmission (265). An attempt to cure one case by parenterally administered α-tocopheryl acetate was unsuccessful. Sheep myopathy has similarities to dermatomyositis, muscular dystrophy, and the myotonic dystrophies of man (266). Walton & Adams (267) note, however, that similarity of muscle fibre change does not necessarily imply a common aetiology, "as a muscle can only have a limited repertoire of response to a variety of noxious agents."

Vitamin K

Two methods for the determination of menadione (2-methyl-1,4-naphthoquinone vitamin K) have appeared (268, 269, 244). Both are modifications of the method of Novelli (270). Reduction of the menadione-protein complex of blood with stannous chloride permits its estimation (244), and chromatography enables a separation from other quinones (269). A method for the estimation of coumarin has been reported (271).

Blood clotting.—Naeye (272) has confirmed earlier work (273) that vita-

min K deficency affects not only prothrombin and proconvertin (synonyms: Factor VII, cothromboplastin, stable conversion factor, convertin) but also plasma thromboplastin component (synonyms: Christmas factor, platelet cofactor II, β-prothromboplastin, PTC). Serum levels of PTC in humans were depressed by 4-hydroxy-coumarin compounds and also in patients with vitamin K deficiency associated with pancreatic fibrosis. Vitamin K analogues restored PTC activity (273). Shanberge (274) studied the postulate that the prolonged prothrombin time induced by coumarin derivates is due to a decrease in proconvertin activity. He concluded that prolongation of the prothrombin time does not reflect a true hypoprothrombinaemia. He postulated a factor other than proconvertin, necessary for the conversion of prothrombin to thrombin. The role of phosphatidyl-ethanolamine and phosphatidyl-serine in blood coagulation (275, 276) is not discussed here.

Anticoagulant therapy studies have been reported (277, 278). The restoration, by vitamins K_1, K_2, K_3, and synkavit, of prothrombin and proconvertin in dogs with chronic bile lack has been studied (279). Chicks given sulfaquinoxaline require more of fat soluble vitamin K substitutes than of water soluble substitutes. Possibly massive therapy with sulpha drugs interferes with bile secretion (280).

The metabolism of prothrombin has been studied following administration of C^{14}-glycine to normal dogs. The turnover rate of the prothrombin greatly exceeds that of the plasma proteins, but the estimated turnover times are smaller than those deduced from the response times of prothrombin when vitamin K deficiency is corrected (281).

Enzyme relationships.—The hypothesis of Martius & Nitz-Litzow (282, 283, 284) that dicoumarol uncouples oxidative phosphorylation in the respiratory chain by competitive inhibition of a vitamin K-containing enzyme has been criticised. Chance & Williams (285), doubt that dicoumarol affects the single phosphorylating site where Martius suggests vitamin K can act. Their spectroscopic studies yield no information on vitamin K, but an effect of vitamin K in phosphorylation efficiency does not require it to function in electron transport. Lehninger *et al.* (286, 287, 288), studying oxidative phosphorylation catalysed by a multienzyme system from rat liver mitochondria, showed that dicoumarol uncouples the phosphorylation linked with ferrocytochrome-*c* oxidation (288). The same concentration of dicoumarol also uncouples the segment of the electron transport chain from substrate (β-hydroxybutyrate) to cytochrome-*c* (287) as well as the overall process from substrate to molecular oxygen, to the same extent (286). Both segments of the respiratory chain are thus equally sensitive, but the phosphorylation coupled to oxidation of ferrocytochrome-*c* should not be affected if Martius is correct. Cooper & Lehninger (288) suggest that the dicoumarol-sensitive enzyme system is not an electron carrier between diphosphopyridine nucleotide and cytochrome-*c* but is a component of the phosphate transferring enzyme system responsible for ATP formation at the three known phosphorylating sites in the respiratory chain (289). This confirms doubts expressed by Chance & Williams. It does not predicate an alternative

nonphosphorylating route for the reduction of cytochrome-*c*. Japanese workers, however, suggest that vitamin K_3 acts as a hydrogen carrier (290).

Brodie & Gray (291) have described cell-free systems from *Mycobacterium phlei* and *Corynebacterium creatinovorans* sensitive to dinitrophenol, in which particulate and supernatant fractions have to be combined to give oxidative phosphorylation with P/O ratios approaching 1.0 (292). Fractionation of the supernatant by Nason's method (293) to give a greatly increased menadione reductase activity, had no effect on the capacity of the supernatant to reactivate oxidation and phosphorylation of the particulate fraction when either succinate or fumarate was the electron donor. This provides further evidence of the involvement of menadione reductase in oxidative phosphorylation.

Intraperitoneal injection of large amounts of menadione reduces incorporation of labelled orthophosphate into labile nucleotide phosphate in liver and heart, and this is associated with a fall in liver glycogen content (294), confirming Martius & Nitz-Litzow (295) that menadione has no intrinsic vitamin K activity. To explain the fact that small amounts of menadione given orally increase the P/O ratios obtained on oxidation of ρ-hydroxybutyrate by liver mitochondria, conjugation of the quinone with a phytyl chain is regarded as a limiting reaction, soon exceeded when excessive amounts of menadione are given (294). Martius (296) gave labelled menadione to chicks and on the basis of the distribution in different solvents of vitamin K from mitochondria he concluded that conjugation of menadione, producing vitamin K_1, occurred.

The distribution of vitamin K in cell fractions has also been studied (297). Beef liver was fractionated and the vitamin was determined biologically. The vitamin K was associated with the nuclear and, more important, the mitochondrial fraction. The molar concentration of vitamin K in the latter was about one third that of cytochrome-*c* and one fifth that of riboflavin. Studies by Martius with labelled menadione also showed that almost all the vitamin K was in the nuclear and mitochondrial fractions of chick tissues (296). It had previously been reported (298, 299) that mitochondria contained little or no vitamin K. The concentration of 0.38 μM per gm. nitrogen now reported is more than two hundredfold that reported earlier.

Some 60 to 80 per cent of the dicoumarol in liver from rats treated with dicoumarol is associated with the supernatant phase remaining after removal of mitochondria (300). The action of vitamin K_1 in reversing hypoprothrombinaemia does not appear to depend on any gross displacement of the drug from the liver or its sub-cellular fractions, which remained unchanged. Only 14 to 16 per cent of the dicoumarol was associated with the mitochondrial fraction (300). The gross pathology of coumarin poisoning has been described (301).

Arnon and collaborators (302, 303) have studied multienzyme systems of chloroplasts which can be divided into three groups which control the

three phases of photosynthesis, namely, photolysis of water, photosynthetic phosphorylation, and carbon dioxide fixation. In the second phase, vitamin K is classed as an electron carrier and placed in the "electron ladder" between flavin mononucleotide and ascorbate. Carbon dioxide fixation is inhibited by vitamin K_3 and it is suggested that its addition diverts the flow of hydrogen from carbon dioxide fixation to photosynthetic phosphorylation (302). Antimycin A has no effect on photosynthetic phosphorylation, suggesting that the antimycin A-sensitive factor, which may or may not be identical with the lipid cofactor which tocopherols can replace (304), is not necessary. Nason & Lehman (211) were unable to find in green leaves their lipid cofactor which activates diphosphopyridine cytochrome-*c* reductase.

Haemolytic anaemia.—The association of haemolytic anaemia and kernicterus in infants with overdosage of vitamin K substitutes (305, 306) has been further studied. The incidence of kernicterus in babies has been related to quantities of vitamin K substitutes given (307). Intramuscular injection of 'synkavit' [tetra-sodium-2-methyl-1,4-naphthoquinone-diphosphate (308)], even in full term infants (308) and in the premature infant (309), resulted in increases in serum bilirubin. Poor liver function may be involved in the hyperbilirubinaemia of the newborn, or there may be excessive haemolysis of erythrocytes in which the therapeutic use of oxygen is an aetiological factor, but reduction of therapeutic intramuscular doses of vitamin K substitutes in infants from 30 mg. per day to 1 mg. per day or less is desirable (310). The sensitivity of the premature infant is probably associated with its low vitamin E status. It has been shown that in vitamin E-deficient rats haemolysis and haemoglobinuria can be produced by intra-muscular injection of several vitamin K substitutes (311, 312) but not by vitamin K_1, and that α-tocopherol will prevent haemolysis and haemoglobinuria even when given 4 hr. prior to the injection (312). Diphenyl-*p*-phenylenediamine is also an effective preventative (312). Synkavit does not produce haemolysis of erythrocytes *in vitro* and the haemoglobinuria appears to be secondary to renal tubular damage induced by vitamin E deficiency (311). Indeed, small amounts of 2-methyl-1,4-naphthoquinone and its salts added *in vitro* appear to protect the erythrocytes of new born infants against hydrogen peroxide haemolysis (313). The involvement of SH groups in these phenomena has already been suggested.

In rats, massive doses of α-tocopheryl hydroquinone cause a haemorrhagic syndrome against which menadione can provide protection. Possibly the hydroquinone acts as an antivitamin with an antivitamin/vitamin ratio of 700:1 on a molar basis. A depression of plasma prothrombin not controlled by menadione occurred in muscular dystrophy patients given α-tocopherol hydroquinone (314).

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NUTRITION[1,2]

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Introduction

For practically all of the first half of this century, during which nutrition has emerged as a science, problems of deficiency disease and undernutrition have been emphasized. Bigger animals and men and faster growth have become almost synonymous with good nutrition. Public nutrition programs have been dedicated largely toward increased consumption of milk, meat, eggs, fresh fruits and vegetables, whole grain and enriched cereals, and practically everything in the usual diet. There have been warnings against gluttony since time immemorial, but until very recently overconsumption of foods was not a major concern of the nutritionist. Some of the programs and recommendations have been based as much upon faith as upon scientific evidence. It has not been unusual in the interpretation of dietary surveys to assume that the lower third or half were "deficient" because they consumed less than the average intake. The levels of intake recommended as desirable have been largely determined by opinions as to how much the "allowance for safety" should be. This is clear since with the same basic evidence available, various competent groups (1 to 4) have arrived at different recommended levels. The Food and Nutrition Board (1) has explained the differences in philosophy between the American, Canadian, and British recommendations. Their discussion and indeed the term "allowance for safety" is predicated upon the idea that excess is preferable to limitation.

Shortly after the last war the increased risk to health associated with obesity was publicized. Although the causes of obesity are still obscure, the methods of treating obesity are obviously unsatisfactory, and the benefits which may be derived from reducing diets have not been clearly demonstrated. New and fabulous reducing diets are introduced weekly and at the same time the public is urged to eat bigger and better breakfasts and lunches. Practically all of the available foods are propagandized either upon the basis that they contain very few nutrients or very many. Both the public and the nutritionists are confused over the relative importance of the things they are taught or have to teach. For example, in a recent survey of school children (5) a sizable group was found to be overweight and another group was consuming less than recommended amounts. These data reveal that the intake of practically all of the essential nutrients is positively correlated with the calorie intake. Apparently the big eaters generally eat more of everything. Thus, to a greater or lesser extent, the same group of children are commended for their large nutrient intake and condemned for being too fat.

[1] The survey of the literature presented here was completed in November, 1956.

[2] The authors wish to express their sincere appreciation to Miss Ann Peterson for her valuable assistance in the preparation of the manuscript.

Of course, it is possible to eat the recommended amounts of all nutrients without consuming too many calories but food habits being what they are, it is worthwhile considering how much responsibility the nutritionist has for the incidence of obesity in this country.

Shortly after or coincident with the development of interest in obesity, a major interest in the role of nutrition in various degenerative diseases has developed. Atherosclerosis, diabetes, and various other diseases (6, 7) appear to be largely those of upper income or sophisticated groups. The evidence that "good diets" by past definition are an important causative factor is so strong as to be nearly conclusive although factual information upon the role of various materials in such diets is only beginning to be accumulated. Since these diseases are the major cause of death in much of the world, they must be a principal concern of medical research, including nutritional investigation, in the future. The nature of chronic disease makes the experimental investigation difficult. Work in applied nutrition can also be expected to be more difficult in the future since promoting the consumption of customary foods is much easier than their restriction. In the future we believe that optimum nutrition will be defined for each of the nutrients as an increasingly narrow range between deficiency and excess, and that these will undoubtedly depend upon body function and environmental conditions. In the solution of these and other nutrition problems and in the application of the findings, the nutritionist must bear in mind that he is responsible not only for seeing that people eat what they should, but that they can eat food that they enjoy.

In this review no attempt has been made to cover the "nutrition literature." Attention has been focussed on conditions which might be related to "overnutrition," even though we realize that such a designation may simply be an expression of ignorance. Some of the classical deficiency diseases were at one time considered due to overconsumption of certain foods, such as corn and white rice. There is considerable justification for this point of view, of course, and atherosclerosis, diabetes, obesity, etc., may turn out to be similarly classifiable.

Atherosclerosis

Evidence continues to accumulate implicating nutritional factors in the problem of atherosclerosis and with its sequelae in man, thrombo-occlusive disease of the coronary, cerebral, renal, and peripheral vessels. Four principal approaches have been followed: (*a*) epidemiologic, attempting to correlate in national and international studies dietary relationships to atherosclerotic heart disease and various lipide components in serum; (*b*) clinicopathologic, studies of humans under controlled conditions, largely aimed at establishing those factors which affect the levels of the various serum lipide components; (*c*) studies of the development of vascular lesions in experimental animals; and (*d*) basic studies of lipide metabolism, largely in experimental animals. Although this classification of the work has been used in the organization of the present discussion, it is not entirely satisfactory.

Not only are the studies interrelated, but the degree of atherosclerosis can only be determined at autopsy. This is seldom practical on the same subjects involved in nutrition studies. It has been necessary to use less direct measures such as the level of serum cholesterol or certain lipoproteins and to assume that these provide adequate indication of the extent of atherosclerosis, or to use vital statistics collected from various sources and assume that these are representative of the groups whose diet is studied. The disadvantages of such unavoidable maneuvers are readily apparent and will be discussed more fully below. The data may generally be assumed to be correct. The conclusions and associations made by various authors, including ourselves, may not.

It should be pointed out that most investigators believe that atherosclerosis is a condition of multiple etiology. This has been amply pointed out in many reviews [e.g., (8)]. In this discussion we deal largely with those reports relating to nutritional studies, but in so doing we do not wish to imply that atherosclerosis is uniquely a nutrition problem.

Epidemiologic Studies.—Numerous isolated reports during the past 25 years have called attention to the relative absence of atherosclerosis in certain socioeconomic and racial subgroups (9). The beginning of the present period of systematic epidemiologic studies of the problem was marked by a series of reports (10, 11, 12) which showed that the incidence of fatal coronary artery disease was reduced in Norway during the period of the German occupation and in the period immediately following. Morris (13), as well as others, however, have pointed out that the pattern of disease rates in Norway had begun to revert toward prewar levels before the end of the war and considerably before the prewar food supply had been re-established. Keys *et al.* (14) have for several years been impressed with a close correlation between the total fat intake and the incidence of coronary artery disease. Keys and his associates, by studying dietary patterns, serum lipides, body fat, and disease incidence rates in numerous national populations including various socioeconomic subgroups have developed the following picture: coronary artery disease and serum cholesterol levels are highest in those populations in which the intake of total fat is highest, whereas other factors such as dietary cholesterol, total calories, the incidence of obesity, and the racial background per se are not closely correlated with evidence of coronary atherosclerosis. The epidemiologic studies during the last year or more have been primarily concerned with verifying and systematizing the hypothesis of Keys and with investigating other variables in these populations in the hope of finding other factors which have a greater correlation, and presumably more important causal relationship with the incidence of atherosclerosis.

Bronte-Stewart *et al.* (15) presented a study of 364 men aged 40 to 58 years from Capetown. The subjects included persons of three major racial classifications and included a wide range of economic status. The serum cholesterol and cholesterol in the beta-lipoprotein fraction (electrophoretic) were determined. A stepwise increase in serum lipide levels, with the Bantus

lowest, Cape-colored intermediate, and Europeans highest, was observed. A similar relation to economic status was also noted within racial groups. There was an excellent positive correlation of the serum lipide picture with the intake of total fat and total animal fat ingested. No correlation with the ingestion of vegetable fat was found. On the basis of registered deaths in Capetown, Bronte-Stewart *et al.* state that the incidence of death secondary to coronary artery disease is twice as great among Europeans as among Cape-colored and that coronary artery disease in the Bantu is very rare. The great difference in incidence of coronary disease among these racial groups was confirmed by studies of electrocardiograms of 5004 persons from the area (16) and by an earlier report of Higginson and Peppler (17). Bersohn and Wayburne (18) determined the serum and cord blood cholesterol concentration in 37 European and 51 African mothers in a Johannesburg hospital. Although the mean serum cholesterol for the European mothers was 258 mg. per 100 ml. compared to 190 mg. per 100 ml. for the Africans, the mean serum cholesterols for the fetuses were nearly identical.

Kimura *et al.* (19) have analyzed 10,000 post-mortem examinations in Japan and reported the incidence of sclerosis of the aorta, coronary arteries, and the basilar artery according to sex, place of habitation, work and use of alcohol and tobacco.

Scrimshaw *et al.* (20) studied the serum cholesterol and β-lipoprotein levels of 99 men and women in a rural zone of Costa Rica, and compared the findings with those obtained in the United States and in rural Guatemalans (21). At all ages the Costa Rican subjects had mean cholesterol values slightly higher than rural Guatemalans but much lower than the North Americans. No consistent increase of serum cholesterol levels with age was noted. The S_f12–20 and 20–100 β-lipoproteins were only slightly lower than those found in North Americans. The S_f0–12 levels were much lower, sufficiently so to account for most of the differences in serum cholesterols observed between Costa Ricans and U. S. subjects. Since the Costa Ricans, in contrast to the Guatemalans, were largely of European origin, these differences were considered as likely due to environmental rather than genetic factors. In a study of the lipoprotein and cholesterol concentrations in sera of Nigerians Mann *et al.* (22) observed that the mean serum cholesterol of 46 Nigerians was 130 mg. per 100 ml. compared to a value of 213 mg. per 100 ml. for an age- and weight-matched group of United States citizens. Although these workers observed a lower dietary fat intake in the Nigerians, they suggested that a relationship of muscle mass and energy expenditure to serum lipides might be more pertinent.

The dangers implicit in making too sweeping conclusions concerning the relationship between the incidence of coronary disease and dietary fat have been emphasized (23). On the basis of vital statistics and data on country-wide food supplies, if only the United States, Italy, and Japan are considered, there is a good correlation between the incidence of fatal coronary artery disease and the national *per capita* consumption (disappearance) of calories,

total protein, animal protein, total fat, vegetable fat, per cent of calories from total fat and from animal fat. If, however, the data from a number of other western European countries and Canada, Australia, and Finland are included, the correlation no longer holds. Apparent consumption of total fat and animal fat is similar in countries where the incidence of fatal coronary artery disease is very different. Italy, for example, with a low *per capita* intake of total and animal fat, has a greater incidence of fatal coronary disease than do Sweden, Norway, and the Netherlands. The possible differences in recording and reporting of disease in various countries must be considered. There are also obvious objections to the use of crude data on national food supplies. In 1950 the age-adjusted death rates for coronary heart disease for white males and females were roughly twice as high in some states as in others (24). Whether such differences are explicable upon a dietary basis remains to be demonstrated.

Other recent work has failed to explain the different incidence rates between racial groups on the basis of dietary habits. Epstein *et al.* (25) compared the dietary habits and incidence of atherosclerosis in Jews and Italians in the United States. Although the criterion of roentgenographic evidence of aortic calcification was relied on heavily, a significant correlation of dietary fat intake and incidence of atherosclerosis was observed in men but not in women. The mean caloric and total fat intakes were similar for the two racial groups, although the Jews had somewhat greater fat intakes. The rate of coronary artery disease was two times greater in Jews than in Italians, a difference that could not be attributed entirely to dietary factors. Page *et al.* (26) conclude that the lower serum lipides in Navajo Indians, as compared with a control group from Cleveland, is best explained on a hereditary basis since dietary habits were not believed to be significantly different. Darby *et al.* (27) in a larger series found mean levels of serum cholesterol in a Navajo population that were essentially indistinguishable from "normal" levels in the United States. These findings are of interest since the diet is said to be intermittently generous in fat (mutton), but the incidence of coronary disease appears to be low among the Navajo (28).

Keys *et al.* (29) re-evaluated the importance of physical activity in populations differing in serum cholesterol values. Although they could not rule out the influence of physical exercise on serum cholesterol levels, they considered other factors, particularly the level of dietary fat, to be of greater importance. In another study Keys *et al.* (30) indicate that, based on a rather wide range of cholesterol intakes by subjects in Minnesota and on Sardinia, there is no relationship between the ingestion of cholesterol and serum cholesterol levels in man.

Clinico-Pathologic Studies in Man.—Many systematic studies of the effect of feeding purified diets and dietary supplements to humans on the levels of various serum lipide components have been completed during the past year. The importance of these measurements rests upon two assumptions: (*a*) that the laboratory determination correlates with the severity of

atherosclerosis and (*b*) that the introduction of a dietary substitute or supplement which "favorably" affects the serum lipide measurement does, in fact, act as a prophylactic or curative factor in the disease.

The general theory of Gofman and his associates (31) that certain lipoproteins of the serum are elevated when atherosclerosis is most severe and are causally related to the development of the disease is well known. They have evolved an Atherogenic Index,

$$\text{A.I.} = 0.1\ (\text{standard } S_f 0\text{-}12) + 0.175\ (\text{standard } S_f 12\text{-}400)$$

with the standard S_f lipoprotein concentrations expressed as mg. per 100 ml. of serum (32). The equation was derived by linear discriminant analysis and the weighting provides the best discrimination between a population with myocardial infarction and a matched population without clinical coronary disease. The correctness of the equation thus rests upon the characteristics of the populations used in its development, and as more information has become available, the equation and even its parameters have varied (33).

The results of the first large independent test of the hypothesis have been published (32). The serum lipides were measured upon approximately 15,000 persons over a three-year period in four different laboratories. Of this group there were 4,913 men between 40 and 59 years of age who were judged to be clinically normal and 82 of this group developed evidence of new disease during the two-year follow-up. The people studied came from various walks of life but cannot be assumed to be representative of the population of the United States.

Much of the report is devoted to methodology, but, most important, the cooperating groups fail to agree on the interpretation of the data. One group concludes that "there exists a predictive relationship of various blood lipide measures with *de novo* coronary disease" and emphasizes the superiority of standard $S_f 12$–20 lipoproteins and the measured Atherogenic Index in predicting new cases. The other group states that while atherosclerosis, as manifest by evidence of coronary artery disease, is associated with an antecedent elevation of the serum $S_f 20$–100 (and possibly of $S_f 12$–20) lipoproteins and serum cholesterol, this elevation "was not of clinical use in predicting those individuals who would develop coronary heart disease." Furthermore, "the use of $S_f 12$–20 and 20–100 lipoprotein measures, or the related A.I. had no advantage over the simpler measurement of cholesterol in the characterization of the men prone to develop coronary heart disease." While the reasons for these divergent interpretations cannot be adequately discussed here, it seems an inevitable conclusion that when competent people fail to agree on the interpretation of the same data, the value of serum lipide measurements in predicting coronary heart disease must be considered as doubtful at the present time. One of the groups points out that "the difficulty of demonstrating an association of the serum lipides with atherosclerosis may be related to the inappropriate use of an American population which is almost universally affected with atherosclerosis." It can scarcely

be doubted that had the population studied consisted of Guatemalans, Bantus, and Americans with the serum lipide characteristics discussed in the previous section, a much better correlation between various lipide measures and coronary heart disease would have been apparent. The measurement of serum cholesterol is certainly far simpler than the measurement of serum lipoproteins, and this alone appears to provide, at least in most populations so far studied, a broad classification as to the extent of atherosclerosis. The prediction of coronary heart disease is obviously much more difficult. within a group with generalized atherosclerosis, such as the major part of the population of the United States. However, this is the most important problem confronting the workers in this country.

Other serum measurement which may have a bearing on atherosclerosis are bile acids (34, 35), unesterified fatty acids (36, 37), lipoproteins and lipoprotein composition by electrophoretic techniques (38, 39), and polyunsaturated fatty acids (40, 41). Two serum transaminase systems (42) have been shown to have value in diagnosing a myocardial infarction.

The reports of Groen *et al.* (43), Kinsell *et al.* (44), and Ahrens *et al.* (45) showed the serum cholesterol levels were lowered by diets containing only vegetable fats as compared with those containing predominantly animal fats. One of the more interesting new techniques which has been applied to this problem is the use of formula diets in the form of emulsions as the sole source of calories for humans in which a particular fat is being evaluated. This technique has been used extensively by the groups associated with Ahrens and with Beveridge (46). Beveridge *et al.* (47) reported on their relatively short-term evaluation of several fats and combinations of fats in a series of medical students and staff at Queen's University. The transfer of subjects from a formula diet based on corn oil to diets based on one of several animal fats resulted in an increase in serum cholesterol levels. Transition from the usual free-choice diet to formula diets containing 60 per cent of calories in the form of butterfat led to no change, whereas corn oil formulas resulted in profound drops in the serum cholesterol. Changes from free-choice diets to fat-free formula diets resulted in decreases in the serum cholesterol which were less marked than those obtained with corn oil. They concluded that corn oil contains a cholesterol depressant factor while animal fat contains an augmenting factor. The longer term studies of Ahrens *et al.* will undoubtedly give a clearer picture of the significance of this and other similar reports.

Bronte-Stewart *et al.* (48) present similar data on 6 Bantu with low serum cholesterol values and 2 Europeans with high serum cholesterol values. Sunflower seed oil and an unsaturated fraction of pilchard oil consistently depressed serum cholesterol and beta-lipoprotein cholesterol levels when fed alone, with a supplement of cholesterol or with some animal fat. They emphasized the proportion of unsaturated and saturated fatty acids as the significant difference in the two groups of oils.

Herbst, Lever & Waddell (49) continued their study of the effect of intravenous infusion of oil emulsions on serum lipides. They reported that the

change in lipoproteins obtained by infusion of fat is similar to that obtained by heparin administration. Sinclair (50) has written a provocative statement which proposes the hypothesis that a proper ratio of linoleic acid to other fatty acids, as outlined by Holman (51) and others in animal studies, and the pyridoxine intake may be critical to the problem of cholesterol metabolism in man and the development of atherosclerosis.

Low fat diets have been used for controlling the level of serum lipides in medical practice (52). Roen (53) has reported on the reduction of serum lipides in 50 hypercholesteremic patients maintained on low fat diets and followed 2½ to 6 years on an outpatient basis. Serum total cholesterols were lowered a mean maximum of 146 mg. per 100 ml. and 82 per cent of the maximum fall was maintained throughout the study.

Although Keys *et al.* (54) found that alimentary hypercholesteremia was lesser in degree and duration in active men than in sedentary men, the role of activity and weight change is debatable (54a, 29). Moore *et al.* (55) reported that in contrast to a similar study with women 24 men losing weight showed a drop in serum cholesterol and total lipides. Life insurance statistics emphasize the increasing incidence of coronary heart disease (56). The importance of obesity, particularly in the younger age groups, is emphasized but considerable contrary data exist (8).

The usefulness of phytosterols in the reduction of serum lipides in man has been further explored (57 to 62). Most reports indicate that the administration of large doses of beta sitosterol gives at least transient reductions in serum cholesterol. Many patients, however, are unable to tolerate for long periods the large doses necessary for effective responses. The lipotropic agents, choline and inositol, are without effect on serum cholesterol levels or on the symptoms of coronary disease in man (63). Although there is evidence (64) that up to half of fatal coronary heart disease is unassociated with the presence of a coronary thrombus, a thrombus formation is often the incident that makes coronary atherosclerosis serious for man. Evidence (65) that the physiologic plasma-clearing mechanism is identical with the heparin activated-blood-clotting inhibiting factor is also accumulating. Thus, the relationships between dietary factors and the clotting mechanism are of importance. Morris (13) ably points out the neglect with which the blood-clotting aspect of thrombo-occlusive coronary disease has been dealt. Fullerton (66) showed that macroscopic alimentary lipemia is associated with an increase in the coagulability of the blood. He claimed such lipemia always occurs after a meal containing 85 gm. of fat but seldom occurs after a meal containing less than 30 gm. of fat. Lasch & Schimpf (67) also found that the ingestion of 70 gm. of butter by normal fasting subjects was regularly followed by a shortening of the blood coagulation time. The maximum effect was noted 120 to 150 min. after ingestion and approximately 30 min. after the maximum lipemia. Kuo & Joyner (68) have concluded that anginal pain after meals is causally related to the level of serum lipides.

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have appeared during the past year. The following will be a consideration of new work related to the production of vascular lesions in which dietary factors seem significant; no critical attempt will be made to evaluate the nomenclature used to describe the lesions produced.

The rat which has been most carefully defined of all experimental animals, as to its nutritional requirements, was considered by many to be highly resistant to the production of atherosclerosis. Hartroft *et al.* (69) have produced a vascular sclerosis resembling the Mönckeberg type in humans by feeding diets low in choline. Wissler (70), who fed a synthetic diet based on lard and cholesterol with superimposed hypothyroidism, deoxycorticosterone acetate (DOCA), and saline drinking water produced lesions in the rat bearing some resemblance to human atherosclerosis. At about the same time atheromatous-like lesions were produced by injecting cholesterol suspensions into doubly ligated femoral arteries [Malinow *et al.* (71)]. Bragdon & Mickelsen (72) produced focal atheroma in the rat by intravenous or intraperitoneal injection of lipoproteins from rabbits fed cholesterol. Intimal Sudanophilia was also produced by feeding rats diets including 30 per cent lard, cholesterol, cholate, and thiouracil. Moskowitz *et al.* (73) used a system of grading the lesions produced in the coronary arteries and aorta to evaluate the effect of sex hormones on atherosclerosis. A detailed microscopic evaluation of "normal" rat vessels indicates that occasional spontaneous lipide-bearing and fibroblastic lesions occur (74). Fillios *et al.* (75) produced gross atherosclerosis in rats by feeding purified diets including cholesterol, sodium cholate, and thiouracil for periods of up to one year. Microscopic coronary artery lesions, associated in one case with a myocardial infarction, were observed. Hegsted *et al.* (76) studied serum lipides in rats fed different levels of cholesterol and cholic acid and different types of fat. The serum cholesterol response of rats were increased markedly by increasing the level of cholic acid and increased slightly by raising the level of dietary cholesterol. There was a general, but not strict correlation of serum cholesterol response with the saturation of the dietary fatty acids. The extent of vascular lipide infiltration was closely proportional to the mean elevation of serum cholesterol, regardless of how the elevations were produced. A similar close correlation of vascular Sudanophilia with the elevations of serum cholesterol, in rats made hypercholesteremic by feeding cholesterol and cholic acid and in which the cholesteremic response was varied by castration and injection of sex hormones, was observed by Fillios & Andrus (77). Wilgram *et al.* (78, 79) observed that dietary cholesterol aggravated the vascular lesions associated with choline deficiency. Endothelial fibroplasia, smooth muscle and internal elastic lamellar fragmentation, and myocardial necrosis were produced in rats fed high levels of calcium, phosphorus, and vitamin D [Gillman & Gilbert (80)].

The rhesus monkey has proven most useful in studies of experimental vascular disease. Mushett & Emerson (81) have expanded the original observations of Rinehart & Greenberg (82, 83) relating a type of intimal fibroplasia to pyridoxine deficiency. Lesions were produced in both monkeys and dogs. The foam cell-lipophagic component with intracellular imbibition of

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lipide are not characteristic of pyridoxine deficiency, per se, nor could lipide infiltration of the intima be superimposed upon the lesions of pyridoxine deficiency through the addition of 2 per cent cholesterol to the diets. Mann & Andrus (84) produced very large elevations of serum cholesterol and beta-lipoproteins and plantar, palmar, and tendinous xanthomata and extensive atherosclerosis by feeding diets containing more than 5 per cent cholesterol for nearly four years to a single rhesus monkey. Part of the cholesterol was supplied as egg yolk. The aorta and its major branches were extensively involved with elevated confluent plaques. The coronary arterial lumina were greatly reduced in size as a result of the intimal involvement. Several raised Sudanophilic lesions were observed in the basilar artery. The majority of the lesions of this animal appears on the basis of gross distribution and histologic considerations to show the closest approximation of human atherosclerosis yet seen experimentally. Andrus *et al.* (85) have presented a comparative histologic study of atherosclerosis produced in the rat by feeding cholesterol, sodium cholate, and thiouracil (75), in the cebus monkey by feeding cholesterol with a soy protein (86) or cholesterol and cholic acid with casein (35), in the rhesus monkey, in the rabbit by feeding cholesterol, and in infant and adult humans. They emphasized the importance of smooth muscle activity in early and late lesions of all the species studied.

The rabbit, the animal first used in studies of experimental atherosclerosis, has been further studied. Rannie (87) has summarized some of the facts of half a century of research that has led to the hypothesis that atherosclerosis is a disease of storage of a relatively inert material, cholesterol. It was emphasized that the thrombotic component of thrombo-occlusive disease is not present in the rabbit. Fillios *et al.* (88) have pointed out some of the pitfalls to previous studies using rabbits in atherosclerosis research. They found that 240 mg. of cholesterol per day (much less than the quantities usually fed in previous studies) was the dosage producing the most discriminating hypercholesteremia (and presumably, atherogenic) response. They also reemphasized the great individual differences in responsiveness of rabbits. These differences were thought to be largely sex-determined but also characteristic of the individual animal. Steiner & Dayton (89) produced hypercholesteremia and early atherosclerosis in rabbits by feeding diets high in vegetable fat. Kritchevsky *et al.* (90) confirmed their original observation that atherosclerosis in the rabbit appears to be more severe when cholesterol is fed in the crystalline state than when suspended in oil. Hirsch & Nailor (91) used rabbits as an assay animal to evaluate further their theory that the deposition of lipides in the aorta depends upon the presence of an insufficiently high ratio of "solvent" (fat that is liquid at body temperature) to "solute" (lipides that are solid at body temperature). They observed that the hyperlipemia and the incidence of atheromata are greater in rabbits which are force-fed cream and added cholesterol than when cream is fed alone; however, the most severe regimen was cholesterol without cream (very high "solute:solvent ratio"). Loewi (92) describes a lesion consisting of areas of necrosis, calcification and fibrosis, mainly of the media, in guinea

pigs and rabbits associated with feeding of diets deficient in vitamin E. Rinzler *et al.* (93) presented a method for the detection of coronary atherosclerosis in the living rabbit. Ergonovine, a sympathomimetric drug, was injected and the electrocardiogram was examined for evidences of abnormality in the S-T component. Dihydrocholesterol and soy sterols are absorbed in significant quantities by rabbits (94). Furthermore, the soy sterols resulted in the induction of atherosclerosis. Such studies, as well as others discussed later, may cast some question upon the wisdom of using sitosterols for reducing serum cholesterol levels.

The textbook of Katz & Stamler (95) adequately documents the susceptibility of the chicken to experimental atherosclerosis and describes the way in which these workers and others have used the chicken in various studies. Nichols *et al.* (96) reported production of atherosclerosis in the bird by prolonged feeding of dihydrocholesterol. The production of atherosclerosis in the thoracic aortae of the parakeet by feeding 1 per cent cholesterol and a seed diet has been reported (97). Severe aortic arteriosclerosis characterized by intimal thickening, medial degeneration, and calcification in an elephant has been reported (98). This animal had been maintained for several years in a zoo on a diet of oats, hay, bread, lettuce, carrots, and an undetermined level of peanuts. Death was ascribed to cardiac failure secondary to pronounced arteriosclerosis of many small coronary arterial branches.

These reports re-emphasize the probability that many different vascular defects can be produced in experimental animals by different dietary regimens, although there are great differences in susceptibility among species. Those lesions which most resemble human atherosclerosis are associated with elevated levels of serum lipides. Since the effects of certain dietary constituents, particularly the fat, on the serum lipide composition of man and of certain experimental animals is similar, considerable optimism as to the usefulness of studies of experimental atherosclerosis is warranted.

Basic Nutritional Studies in Animals Relative to Cholesterol Metabolism.—The relationship of dietary fat, particularly its fatty acid components, to the metabolism of cholesterol in animals has received extensive consideration (e.g., 99, 100, 101). Our knowledge of the essential fatty acids in animal nutrition (102, 103) is based largely upon their activity in relation to growth, prevention of dermal lesions, and in pregnancy and lactation in rats. Peifer & Holman (104), using the time of development of dermal lesions associated with essential fatty acid deficiency in rats as an assay method, showed that cholesterol feeding or alloxan diabetes accelerated the development of essential fatty acid deficiency.

The biochemical importance of linoleic acid as an essential fatty acid may be related to its probable position as a precursor of arachidonic acid in the animal organisms. New evidence supports the hypothesis that arachidonate is formed by a condensation of linoleate and acetate (105, 106) and that pyridoxine is involved in the conversion (107).

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The metabolism of long-chain fatty acids is, of course, still poorly understood, although new reports of conversions of fatty acids of greater un-

saturation to those of lesser unsaturation and vice versa, *in vivo*, have been reported (108, 109, 110). Sinclair (50) has suggested that the fatty acid composition of cholesterol esters may be determined by diet and that such esters may be turned over at different rates; however, there is scanty evidence to date to suggest that this is true. In fact, until quite recently (111) attempts to define completely complicated mixtures of fatty acids such as those esterified with cholesterol in a biological system have been very restricted due to lack of suitable methods.

It seems clear that the pancreas (112) plays an important part in cholesterol esterification and absorption. A specific cholesterol esterase activity has been reported in the pancreas (113). Duncan & Best (114) concluded that esterification is a critical step in cholesterol absorption, since sitosterol, but not sitosterol esters of long-chain fatty acids, appeared to be effective in lessening the absorption of cholesterol by the rat. However, Swell *et al.* (115) concluded that plant sterol esters are readily absorbed and converted to cholic acid and cholesterol or a cholesterol intermediate. The growth-promoting effect of cholesterol has been amply demonstrated in experimental animals (116, 117). Such observations have been used to support the importance of cholesterol in fatty acid absorption and transport. Studies of pantothenic acid-deficient rats on low fat or fat-free diets (118) suggested that the failure to respond to hypercholesteremic regimens might be due to insufficiency of fatty acids for esterification and absorption rather than to depressed liver synthesis of cholesterol as had been previously suggested by Boyd (119). The conclusions of Pihl (120) are in partial disagreement with the above; he has found that the stimulating effect of free fatty acids on cholesterol absorption is not due to the formation of cholesterol esters prior to absorption and that the conditions in the intestinal tract favor hydrolysis of cholesterol esters.

Thomasson has published a series of observations on the biological value of oils and fats (121, 122, 123) in rats. Eighteen fats were evaluated with respect to growth and rates of absorption from the gastrointestinal tract. A significant, but not strict, correlation appeared to exist between the absorption rates of fat and growth. Thomasson divided fats into 5 main groups according to descending rates of absorption. Butterfat was placed in group 1; maize oil, cottonseed oil, beef tallow, coconut oil, soybean oil, sunflower oil, groundnut oil, and olive oil were placed in group 2, while rapeseed, poppyseed, and kapokseed oils comprised group 5.

Benton *et al.* (124) studied the effect of different fats on liver fat deposition in rats receiving diets containing 20 per cent fat and 9 per cent casein. Liver fat in rats fed butter or lard was high, while it was low in rats fed corn oil or margarine. The effect was accentuated when the level of protein in the diet was decreased and when choline was omitted. Resynthesized glycerides from butterfat fatty acids gave the same effect as butterfat; the solid fatty acid fraction caused a much greater accumulation of liver fat than did the liquid fraction; and stearic acid a greater accumulation than oleic acid. The unsaponifiable fraction of butter was without effect.

Swell *et al.* (125) have reported further studies relating the effect of dietary fat and fatty acids on the fecal excretion of a calcium oleate phosphate complex. A fecal "phospholipid" precipitable by acetone was shown to contain 2 atoms Ca^{++}, 1 mole $HPO_4^{=}$, and 2 moles of oleic acid. A series of rats were fed diets containing different natural oils, palmitic, and oleic acids, and the daily excretion of lipide phosphorus was measured. The rats fed oleic acid excreted 12.52 mg. of lipide phosphorus per day whereas none of the other groups of rats had a mean lipide phosphorus excretion of more than 1 mg. per day.

The relationship between total body fat and cholesterol metabolism in experimental animals has received further consideration. Lewis & Page (126) compared the lipoprotein and cholesterol concentrations in "mesomorphic" and "ectomorphic" miniature swine fed the same diet and in which relative weight gain was similar. The "ectomorphic" pigs had lower levels of cholesterol and β-lipoproteins. Thus, a genetically determined condition was shown to be associated with obesity and hypercholesteremia. Such findings are analogous to those of Mayer (127, 128) who has studied a genetically determined condition in mice which is associated with obesity, hyperglycemia, and hypercholesteremia. This condition, as well as that associated with transplanted adrenocorticotropic tumors in mice (129), is associated with increased lipogenesis and cholesterolgenesis. Thus, these two types of obesity fit the pattern described by Mayer as "metabolic obesity." Mickelsen *et al.* (130) reported the production of obesity in rats by feeding high-fat diets.

Although, perhaps, the greatest recent interest with reference to cholesterol metabolism in experimental animals has centered around the relationship to dietary fat and fatty acid metabolism, other studies indicate that many dietary factors may be important. Several investigators (75, 131, 132) have reported that the serum and liver cholesterol elevations in rats fed experimental regimens is inversely proportional to the level of dietary protein. Similarly, it has been claimed (133, 134) that the type of dietary carbohydrate influences the level of biliary excretion of bile acids and the level of serum cholesterol in rats in which hypercholesteremia was induced by cholate feeding. Gallogen®, a camphoric acid ester, previously shown to be a true choleretic agent, has a hypocholesteremic effect on cockerels (134a, 134b).

Reducing Diets and Obesity

Most issues of women's magazines which, fortunately, we do not feel compelled to review, have a new wonder method or reducing diet. The scientific literature on methods of weight reduction is somewhat less voluminous but not much less confusing. For many years high protein intakes were generally recommended when calories were restricted. A few years ago Pennington (135, 136) recommended high fat-high protein diets without any marked restriction of calories. The role of protein remained unchallenged until the recent work of Dole and associates (137) reported considerable suc-

cess with diets low in protein. It would appear that any diet which one would ordinarily not want to eat may be a good reducing diet.

In the diet used by Dole *et al.* (137) the protein was limited to 35 gm. per day but each individual was allowed to regulate his total calorie intake by fat and carbohydrate consumption. Good results were reported in approximately 50 per cent of the patients in a follow-up 3 to 12 months after discharge. It was concluded that protein restriction offers a method of appetite control since upon the prescription of more protein, the voluntary food intake increased. The evidence so far available is perhaps insufficient to indite protein as the determining factor in these studies since the difficulties in producing palatable low protein diets are well known. The mass circulation of recommendations for diets even more limited in protein than those used by Dole *et al.* prompted Jolliffe (138) to consider the data upon protein requirements and point out the possible dangers in the widespread unsupervised use of such diets. This paper emphasizes how low the estimates of the minimum protein requirement are (about 0.5 gm. per kg. body weight on a mixed diet and much lower on high quality proteins), but it also stresses the limitations of present information and the extremely low level of protein in the popularly advertised diets (139, 140).

Supportive evidence for the thesis of Pennington that high fat diets are desirable in reducing has been supplied by Kewick & Pawan (141). Very obese patients (35 per cent overweight) were given diets in which 90 per cent of the calories were supplied by either fat, protein, or carbohydrate for periods of five to nine days. When 1000 calories were supplied per day, the weight losses were largest upon the high fat diet (about a pound per day), about half this on the high protein diet, and there was little weight change on the high carbohydrate diet. From determinations of body water the authors conclude that from 50 to 67 per cent of the weight lost must have been fat. No change in absorption or basal metabolic rates was found. The "insensible weight loss" was higher upon the high fat diet. The work thus presumably demonstrates the oxidation of more calories per day, including body fat in obese patients receiving high fat diets. In spite of the apparent limitations, the data warrant consideration. It may be noted that obesity has been produced in rats simply by feeding high fat diets (130), and Kaunitz *et al.* (142) have reported that weight maintenance in young rats requires 25 per cent less calories on a high fat diet than on a high carbohydrate diet. The energy expenditure in the rat studies would appear to be opposite to that in the obese subjects of Kewick & Pawan. Again the role of activity is unknown.

The easiest way to dispose of these studies is to condemn them upon the basis of the second law of thermodynamics or to conclude that changes in activity must account for the calorie expenditure. Neither of these conclusions necessarily follows and the second law can still be preserved. It is now well understood that only a portion of the energy of foods is made available to the body by the formation of high energy bonds. Using 10 kcal./mole of high energy bond formed (143) about 55 per cent of the total energy in fat and carbohydrate appears to be available for tissue formation and main-

tenance, for work and those functions of the body which require energy. We know little about the factors which control the formation of this useful energy or the efficiency with which it is used, but it is the disposition of the energy rather than the total amount that is of primary interest. Various methods are apparently available for changing the disposition of the total energy. These include the "uncoupling" of oxidative phosphorylation by thyroxine or dinitrophenol; alternate pathways of metabolism involving tri- or diphosphopyridine nucleotides TPN or DPN, to a greater or lesser extent or hydrogen transfer from one to the other, may change the yield of available energy (144); increased pyrophosphatase activity resulting in loss of high energy bonds may occur. Thus, if the substrate itself or its products can uncouple or change factors eventually affecting the yield of available energy, a new parameter enters the field of energy requirements and obesity. [See Lardy (145)].

Other aspects of the obesity problem have been extensively reviewed (127). In a well designed experiment, Bernstein & Grossman (146) failed to find support for the glucostatic theory (127).

Hypercalcemias

An area of particular interest in this laboratory for some time has been the definition of calcium requirements. Whereas it is commonly stated that an inadequate intake of calcium is the most prevalent dietary lack in the United States, calcium deficiency in any diagnosable form is uncommon even in areas where the calcium intake is far less than in the United States (147, 148); and we have questioned the basis for formulating the dietary requirement of calcium (149). We are not aware of any important new advances in recent years for developing an adequate assessment of the desirable calcium intake. It is worthwhile to point out at this time that, at least at the clinical level, there appears to be more evidence of calcium toxicity than calcium deficiency. Since its description in 1949 (150) numerous papers have appeared upon metastatic calcification (Milk Drinkers' Syndrome) associated with high milk and alkali intakes, generally, if not always, in ulcer patients (151 to 155). The calcinosis, renal lesions, etc., are reversible following reduction of the calcium and alkali intake. While these findings may, or may not, have an important bearing upon recommended calcium intakes or the general level of calcium in the United States and similar countries, the possible relation to the development of renal calculi, for example, merits consideration.

Hypercalcemia in infants has apparently increased markedly, and to a different degree, in various parts of Europe in recent years [see review (156)]. The condition is apparently rare in the United States. Morgan *et al.* (157) report that 4.6 per cent (15 children) of the admissions of infants between 6 and 12 months of age to Dundee hospitals were attributable to hypercalcemia and 4 of these children died. In many, but not all, of such infants (158) a history of high or relatively high vitamin D intakes is found. There has been speculation that some infants might be abnormally sensitive to the action of vitamin D (159). However, Morgan *et al.* conclude that high

vitamin D intakes is the most likely cause, probably when associated with high calcium intakes. It is important to note that many of these infants have not had massive doses of vitamin D. Reconstituted National dried milk in the United Kingdom is reported to contain 1400 I.U. per quart and the levels of vitamin D supplementation in milk and infant foods is such that intakes over 2000 I.U. [considered toxic by Jeans and Stearn (160)] can easily be obtained. The question was raised "whether more illness (in the United Kingdom) may now be due to vitamin D than the rickets it is designed to prevent (157)." There is a clear need for a reassessment of the degree of fortification necessary and the relation of vitamin D need to calcium intake.

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X-RAY STUDIES OF COMPOUNDS OF BIOLOGICAL INTEREST[1]

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Decisive advances have been made since this subject was last reviewed in these pages by Corey (1). Six years ago people were still speculating about the structure of biological macromolecules without having solved any one of them. Today the most important of the fibre structures have been solved, and the globular proteins, which seemed so intractable then, are being subjected to powerful new methods of x-ray analysis.

The milestones passed each year make an impressive list. The year 1951 brought the discovery of the α-helix and the pleated sheet as the structural basis of fibrous polypeptides and proteins. The theory of x-ray scattering from helical chains, developed in 1952, led to the solution of the structure of deoxyribonucleic acid in the following year, which also saw the solution of the phase problem in haemoglobin and the calculation of its first Fourier projection. In 1954 came the first tentative results on vitamin B_{12}, the most complex structure so far to be solved by x-ray analysis. In 1955 collagen was solved and the first Fourier projections of myoglobin were obtained; the present year saw decisive advances in the field of crystalline viruses, as well as the complete solution of the structure of vitamin B_{12}. These discoveries will form the main subject of our report. A section will be devoted to "building stones" of proteins and nucleic acids, but space will permit detailed treatment of only a few of the 50 or so organic structures of biological interest which have been solved in the interval since Corey's review (1).

The Use of Heavy Atoms in the X-ray Analysis of Organic Compounds

X-ray diffraction pictures contain only half the information needed to find the positions of the atoms in the unit cell.[2] The other half has to be supplied by human ingenuity or by indirect methods. This is because only the intensities of the diffracted rays are open to direct measurement, whereas determination of the structure also requires knowledge of their phases. What can be deduced from the intensities alone is merely the distribution of interatomic vectors in the unit cell, referred to a common origin. In simple com-

[1] The following abbreviations are used in this chapter: DNA for deoxyribonucleic acid; RNA for ribonucleic acid; and TMV for tobacco mosaic virus.

[2] A short article on the application of x-ray crystallography to the study of biological macromolecules, written by Kendrew & Perutz for nonphysicists, may be found helpful (2). Fuller introductions to x-ray analysis will be found in Bunn's *Chemical Crystallography* (3), and Robertson's *Organic Crystals and Molecules* (4).

pounds of known constitution this "vector structure" or "Patterson" is usually sufficient to work out a stereochemically satisfying arrangement of the atoms, which may then be refined by a variety of mathematical methods. The final result is expressed in the form of contour maps ("Fouriers") which give the distribution of electron density either in projection on a plane or as a series of sections cut at close intervals through the unit cell.

Analysis by trial, on the basis of the vector structure, becomes impracticable with large molecules whose constitution is known only in part. In such cases heavy atoms can often be used to determine the phase angles directly, and to find the chemical constitution of an organic compound by purely physical methods. The presence of heavy atoms in an organic structure has a profound effect on the intensities of the diffracted rays, because the electrons in the few heavy atoms are concentrated at points and scatter x-rays in phase, whereas the electrons of the much more numerous light atoms are distributed over a large volume, so that most of their scattering contributions are lost by interference. The simplest version of the heavy atom method usually depends on a single halogen or metal attached to an organic compound. As a first step in the analysis the positions of the heavy atoms are found from the vector structure. Once this is done the phase angles of the diffracted rays are calculated as though they were determined by the heavy atoms alone. With luck, these approximate phases serve to find the positions of some of the light atoms which are then used in turn to work out more accurate phase angles. From this stage onwards the structure can be refined by a repetitive cycle of mathematical operations using computing machines. It appears that this method of analysis works almost automatically provided the amplitude of the heavy atom is greater than the mean amplitude scattered by all the light atoms put together. In theory, this means for instance that the maximum molecular weight of an organic mercury compound whose structure could be determined directly would be of the order of 1700. In practice, it appears that this figure can be at least doubled, by the use of ingenuity, as was the case in the analysis of vitamin B_{12}.

The second, and more powerful, version of the heavy atom method requires a pair of isomorphous compounds differing by the weight of only one atom in the asymmetric unit: for instance, an iodine replacing a chlorine. The diffraction patterns of two such compounds are similar except for small changes in the intensities of the diffracted rays brought about by the different scattering power of the two atoms. These changes may be used first to locate the heavy atoms themselves and then to calculate approximate phase angles. After that the process of analysis follows the same course as in the case of the single heavy atom compound described above. Assuming perfect isomorphism, theory indicates that the maximum molecular weight of an organic compound whose structure could be determined by replacing a hydrogen by a mercury atom would be of the order of 35,000. This means that the structure of globular proteins could be analysed by this method. Several attempts on these lines are in fact under way, but it is not yet cer-

tain whether the degree of isomorphism will be good enough to work out the structure of proteins of such large molecular weight.

Bijvoet, Peerdeman & van Bommel have shown that heavy atoms may also be used to find the absolute configuration of enantiomorphous compounds (5). By choice of a suitable wavelength of x-rays, they excite the resonance frequency of the K-electrons in the heavy atoms. This excitation gives rise to differences between the intensities of reflexions from opposite crystal faces which allow a decision to be made between a left-handed and a right-handed arrangement of the atoms.[3] Bijovet and his collaborators have used this anomalous scattering effect to determine the absolute configurations of D-tartaric acid (5) and of D-isoleucine (7) and have found Fischer's convention to be the correct one. Recently Peerdeman also worked out the absolute configuration of strychnine (8). An interesting extension of Bijvoet's method, proposed by Pepinsky & Okaya (9), uses anomalous scattering by heavy atoms for direct determination of the crystal structure itself, as well as the absolute configuration of the asymmetric molecules.

Compounds of Small and Medium Molecular Weight

Vitamin B_{12}.—This vitamin was first crystallized in 1948 in the form of red, strongly pleochroic, orthorhombic needles. On drying, the needles lose water of crystallization and shrink slightly in two directions. Chemical analysis showed the dry needles to possess the approximate composition $C_{61-63}H_{86-97}O_{13-20}N_{14}P$ Co. On hydrolysis the molecule yielded cyanide, 1-amino-2-propanol, 5 to 6 moles of NH_3, 5,6-dimethyl-benziminazole-1-α-D-ribofuranoside-(2′ or 3′)-phosphate, and a deep red gum, from which a hexabasic acid of unknown constitution was crystallized. The crystals of this vitamin-B_{12} fragment had the approximate composition $C_{47}H_{66}O_{16}N_4Co$ Cl, and had lost the nucleotide, the propanolamine, and the CN group. In vitamin B_{12} itself this CN group could be exchanged with thiocyanide or selenocyanide. There were also derivatives in which the dimethylbenziminazole was replaced by adenine, 2-methyl adenine, and 5,6-dichlorobenziminazole.

The main derivatives used in the x-ray analysis were the wet and dry crystals of vitamin B_{12}, the selenocyanide, and the hexacarboxylic acid fragment. The x-ray analysis of an asymmetric molecule containing about 180 atoms, of which only 45 had been assigned to chemically known constituents, was an unprecedented undertaking, begun independently by Mrs. D. C. Hodgkin at Oxford and Dr. J. G. White at Princeton. The development of the work has been described in a succession of short notes (10 to 13); a full account is now in press (284).

Vector diagrams of dry B_{12} revealed the positions of the cobalt atoms

[3] An article on this method by Bijvoet, written for nonphysicists, has appeared in *Endeavour* (6).

and showed that they were surrounded by an octahedral array of atoms, among which the CN group and four nitrogens, belonging to what was at first thought to be a porphyrin ring, were immediately recognised. At the next stage, phases were calculated for dry and wet B_{12} and for the selenocyanide compound, on the assumption that these were dominated by the atoms so far recognised, mainly by the cobalt and selenium atoms. The first electron-density maps revealed the nucleotide group, attached to the cobalt by one of the nitrogens of the benziminazole group, and also a large planar group surrounding the cobalt atom. At this stage Mrs. Hodgkin made the crucial discovery that this planar group was not a porphyrin, but a new kind of ring system not hitherto encountered in biological chemistry. This conclusion was confirmed by the x-ray analysis of the hexacarboxylic acid fragment. From this stage on, successive cycles of refinement were carried out on all four crystal structures, greatly assisted by Dr. Kenneth Trueblood who used the automatic computer (S.W.A.C.). Through the refinement, the position and chemical nature of the other atoms in the molecules became clear and many of the water molecules which fit between them in the crystals were also located.

The formula of vitamin B_{12}, derived from the three-dimensional electron density maps, is $C_{63}H_{88}O_{14}N_{14}P\,Co$, and its chemical constitution is as shown in Figure 1. This differs from the formula shown by Pfiffner & Bird in last year's Review (14) by the number of double bonds in the central ring sys-

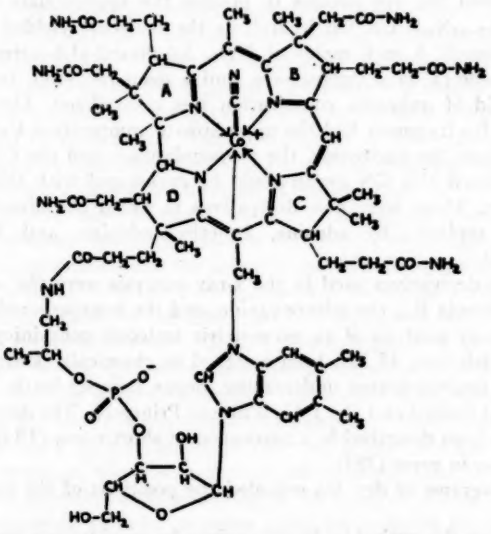


FIG. 1. Formula of Vitamin B_{12}. [Reproduced from Hodgkin *et al.*, Formula I, *Nature*, **178**, 65 (1950).]

tem which is now known to be six and not five, as was believed at an earlier stage. The cobalt atom is seen to be surrounded, approximately in a plane, by four five-membered rings. In three places the rings are linked through intermediate carbon atoms, as in porphyrin, but in one place, between rings A and D, a direct bond between two carbon atoms of neighbouring rings is formed. This is the novel feature which emerged from the electron density maps. The system of five-membered rings forms the centre of the structure to which there are attached eight methyl groups, three acetamide, and three propionamide side chains and a long scorpion-like tail coiling back onto the centre of the molecule. This starts with a propionic acid group linked to a propanolamine which in turn forms an ester-link with the phosphate of the nucleotide, whose benziminazole group links up with the cobalt atom.

The atomic positions found in the crystal of wet B_{12} are shown in Figure 2. The molecule as a whole is not far from spherical. All but one of the side

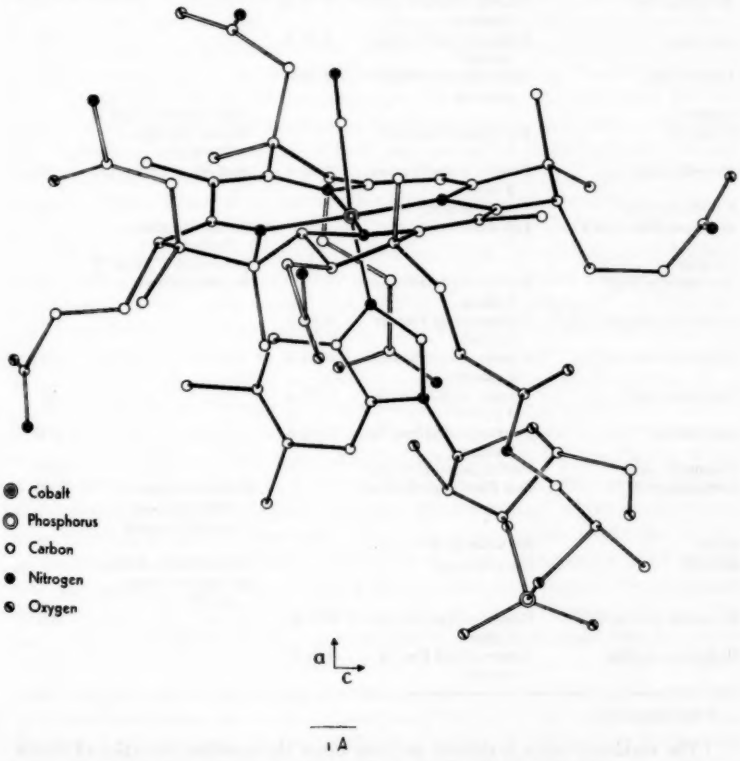


FIG. 2. Molecular structure of dry vitamin B_{12}, seen in projection on the crystallographic b-plane (hydrogens omitted). Drawing supplied by Dr. D. C. Hodgkin.

TABLE I

STRUCTURE OF AMINO ACIDS

Amino Acid studied	Data available	Maximum error of atomic parameters*	Remarks	References
glycine	Projections on 3 planes	0.12 A	Analysis by trial. No Fourier methods	(15)
nickel glycine. $2H_2O$	Fourier projections on 3 planes	0.12 A		(16)
DL-alanine	3-dimensional Fourier analysis	0.02 A		(17)
DL-valine	Cell dimensions only			(18)
DL-leucine	Cell dimensions only			(18)
D-isoleucine	Fourier projections on 2 planes	0.12 A	Absolute configuration determined	(7)
DL-norleucine	Fourier projections on 2 planes	0.12 A		(19)
DL-serine	3-dimensional Fourier analysis	0.02 A		(20)
Lg-threonine	3-dimensional Fourier analysis	0.02 A		(21)
cysteine			See peptides, Table II	
L-cystine	Cell dimensions only		See also peptides, Table II	(22)
DL-methionine	Fourier projections on 2 planes	0.05 A	Analysis of 2 dimorphs	(23)
D-phenylalanine	Cell dimensions only			(22)
DL-tryptophan 2 HCl	Cell dimensions only		See also peptides, Table II	(18)
tyrosine			See peptides, Table II	
Na-thyroxin. $5H_2O$	Patterson projection on 1 plane		No structure yet	(24)
D- or L-histidine HCl. H_2O	3-dimensional Fourier analysis	0.03 A		(25)
DL-glutamic acid. HCl	Fourier projections on 2 planes	0.05 A		(26)
L-glutamic acid	Fourier projections on 3 planes	0.05 A		(27)
L-glutamine	Fourier projections on 2 planes	0.05 A		(28)
DL-aspartic acid	Preliminary report only			(29)
L-asparagine. H_2O	One Fourier projection		Preliminary report only; structure probably wrong	(30)
lysine	No x-ray data			
arginine	No x-ray data		See this text p. 334 for guanidinium compounds	
DL-copper proline. $2H_2O$	Fourier projections on 3 planes	0.12 A		(31)
Hydroxy-L-proline	3-dimensional Fourier analysis	0.03 A		(32) (33)

* See footnote 4.

[4] The maximum error is defined as three times the standard deviation of atomic co-ordinates. If the errors follow Gaussian laws, the probability of any atomic coor-

chains are in the extended (staggered) configuration with reactive amide groups pointing outwards. The benziminazole ring is packed between two of the propionamide side chains attached to rings A and B and is almost normal to the plane of the central ring system. At the stage which the analysis has reached atomic positions are accurate to about 0.3 A, with further refinement still in progress on wet and dry B_{12} and on the fragment.

Amino acids and peptides.—Table I lists the extent of crystallographic information available for each of the amino acids which generally occur in proteins, and also for norleucine and thyroxin. The most accurate structure determinations are those based on three-dimensional Fourier analyses, which include alanine, serine, threonine, histidine, and hydroxyproline. The least accurately known structure, somewhat paradoxically, is that of glycine, which was solved before the general adoption of Fourier methods. On the other hand, the structure of the glycine residue has now been determined very accurately as a component of several peptides (see Table II). Among the newer structure analyses, that of hydroxyproline is interesting as it confirms the *trans* configuration of the carboxyl and hydroxyl groups relative to the five-membered ring, which is nonplanar, the carbon bearing the carboxyl group being displaced by about 0.4 A from the plane defined by the other four atoms; this displacement is towards the side opposite to the carboxyl group. The nitrogen, α-carbon, and carboxyl form a second planar group inclined at an angle to the plane of the ring. The molecule has the *zwitterion* form. These data have proved most useful in the construction of the polyproline and collagen models referred to on pp. 346 and 349.

Histidine is the latest structure to be completed. An analysis of its hydrogen bonding system and accurate determination of bond distances allow predictions to be made about the percentage contributions of different resonance forms to the imidazole ring. This may help us to understand the part played by this ring as an active centre of enzymes and as a link between protein and metal in respiratory enzymes and oxygen carriers.

When one looks through the amino acids with long aliphatic side chains it is interesting to note how very small are the alterations in molecular environment which suffice to change the configuration of the side chains. Thus in D-isoleucine hydrochloride and hydrobromide, two structures with similar unit cell dimensions, it is in fact only the "glycine group" which occupies the same position in both compounds (being planar as usual), while the substitution of chlorine for bromine is sufficient to change the orientation of the isobutyl group by switching it round the C_α—C_β bond. Again, the propionic acid side chain in L-glutamic acid takes up a different orientation from the one found in DL-glutamic acid hydrochloride, although bond distances and

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dinate being in error by three times the standard deviation is 0.1 per cent. In practice, standard deviations are often underestimated and errors may not be strictly Gaussian. As a conservative estimate, the probability of co-ordinates being in error by amounts greater than the ones given in the table may be put at about 1 per cent.

TABLE II

STRUCTURE OF SIMPLE PEPTIDES

Compound	Data available	Maximum error of atomic coordinates*	Remarks	References
α-Glycylglycine	3-dimensional Fourier analysis			(38)
β-Glycylglycine	Fourier projections on 2 planes	0.06 A		(39)
N-acetylglycine	3-dimensional Fourier analysis	0.02 A		(40)
Glycyl-L-alanine	Fourier projections on 2 planes		Preliminary report	(41)
Glycyl-L-tryptophan. $2H_2O$	3-dimensional Fourier analysis	0.03 A		(42)
Glycyl-L-tyrosine. HCl	Fourier projection on 2 planes	0.12 A		(43)
Glycyl-L-asparagine	3-dimensional Fourier analysis	0.03 A	Contains useful table of bond lengths and angles of the peptide group as found in different peptides.	(44)
Cysteylglycine NaI	Fourier projections on 2 planes		y-parameters of atoms may be wrong	(45)
N,N′-diglycyl-L-cystine $\cdot 2H_2O$	Fourier projection on 1 plane and least squares refinement in 3 dimensions	0.06 A		(46)
Glutathione	3-dimensional Fourier analysis		Preliminary report	(47)
Diglycylglycine ethyl ester HCl	Fourier projections on 2 planes	0.2 A		(281)
Triglycylglycine ethyl ester HCl	Fourier projection on 1 plane		Tentative structure	(281)

* See footnote 4.

angles in the two forms are identical. Another example is DL-methionine, which actually occurs in two crystalline modifications differing only in the orientation of the thiomethyl group.

The structure of arginine has not yet been determined, but much interesting work has been done on the guanidinium ion, including analyses of guanidinium bromate (34), methylguanidinium nitrate (35), creatine (36, 37), and creatinine (282). The two analyses of creatine show the existence of several resonance forms, due to three alternative double bond positions in the guanidinium group and two in the carboxyl group. The guanidinium group is planar with a mean C-N distance of 1.33 to 1.34 A, corresponding to slightly less than 50 per cent double bond character. The plane of this group encloses an angle of 85° with the plane of the carboxyl group. Creatinine is reported to be an almost planar molecule, with only the methyl group displaced by 0.25 A from the plane of the other atoms.

At the time of the last review (1), information about the dimensions of the peptide chain was derived from only three structures: N-acetylglycine, β-glycylglycine and diketopiperazine. We now have five more analyses of glycylpeptides, and one of glutathione, all remarkable for their elegance and accuracy (Table II). These structures give a consistent value of 1.32 A for the carbon-nitrogen distance in the peptide link, corresponding to 50 per cent double bond character, and confirm the planarity of the amide group which now forms the basis of all models of proteins and polypeptides. All peptides have the CO and NH groups of the amide in *trans* configuration and the terminal NH_3^+ group in *cis* position with respect to the amide CO. Groups of atoms tend to have the extended (staggered) configuration and to be coplanar even in the absence of double bonds, with the result that the structures of these glycyl peptides can all be defined simply by reference to two or three planes and the angles between them. A particularly valuable piece of information is provided by the structure of N,N′-diglycyl-L-cystine dihydrate: the length of the S—S bond comes out as 2.05 A (single bond distance 2.08 A) and the dihedral angle of the —C—S—S—C— bridge as 101°, which is near the value found in S_8. These data helped Lindley & Rollett to build the cystine bridges in their model of insulin (see p. 352).

Other amides giving useful structural information are urea, whose system of resonating double bonds has been accurately analysed by Vaughan & Donohue (48); nicotinamide (49); succinamide (see 50, where a useful table of bond distances and angles in the amide groups of different compounds can be found); tetraethyl-α-α-dibromosuccindiamide, where the substitution of two ethyl groups on each of the amide nitrogens enabled Corradini & Giacomello to prove the planarity of the amide group more rigorously than has been possible in any other compound (51); and finally succinimide where Mason found an interesting resonance structure (52). Many of the hydrogen bonds in these structures involve charged NH_3^+ and COO^- groups and are therefore not typical of the hydrogen bonds between uncharged CO and NH groups which are so important in proteins. An accurate value for the distance from nitrogen to oxygen in the latter type of bond is found in Bailey's paper on the crystal structure of N,N′-diacetylhexamethylenediamine (119). The distance is 2.88 A, as compared to 2.84 A, estimated by Pauling & Corey for an α-helix with an axial repeat of 1.50 A per residue (108). It will be noted that apart from the tripeptide glutathione, whose analysis is nearing completion, all the peptides studied in detail so far have been dipeptides of glycine. According to physicochemical measurements of polypeptides in solution, at least nine residues are needed to form an α-helix. This shows how great the need is for detailed x-ray studies of still larger peptides of varied composition whose structure could teach us more about the ways of folding to be expected in globular proteins.

Nucleic acid derivatives and related compounds.—Table III, which lists the compounds studied to date, contains a variety of pyrimidine derivatives;

TABLE III

STRUCTURE OF NUCLEIC ACID DERIVATIVES AND RELATED COMPOUNDS

Compound	Data available	Maximum error of atomic coordinates*	Remarks	References
2-Amino-4-methyl-6-chloropyrimidine	Fourier projection on 1 plane	0.12 A		(53)
4,6-Dimethyl-2-hydroxypyrimidine	Fourier projections on 2 planes	0.12 A		(54)
4-Amino-2,6-dichloropyrimidine 5-Bromo-4,6-diaminopyrimidine	3-dimensional Fourier analysis	0.05 A	Positions of H atoms determined approximately	(55)
4,5-Diamino-2-chloropyrimidine	Accurate 2 dimensional Fourier methods	0.08 A	Position of H atoms determined from difference Fouriers	(56)
Uracil	2- and 3-dimensional Fourier methods	0.04 A	Three out of four H-atom positions determined, resonance forms discussed	(57)
Adenine HCl	Accurate 2-dimensional Fourier methods	0.04 A	Accurate determination of H-atom positions and of electron density distribution in ring system. Resonance forms discussed.	(58) (59)
Guanine HCl	Fourier projections on 2 planes	0.12 A	Ten out of 14 possible tautomers ruled out by analysis	(60)
Xanthazole	3-dimensional Fourier analysis	0.03 A		(61)
Cytidine	Fourier projections on 3 planes	0.12 A	Reviewed by Corey (1)	(62)
5′-Bromo-5′-deoxythymidine (2 forms)	Fourier projections on 2 planes	0.2 A		(63)
Adenylic acid b	Fourier projection on 1 plane		Preliminary report	(64)
2′,3′-Isopropylidene, 3:5′-Cycloadenosine iodide	Fourier projection on 1 plane and projections on 1st and 2nd layer line	0.12 A	Direct structure analysis using phases derived from iodine positions	(65)
5′-Iodo-5′-deoxy-O^{2}:2′-cyclouridine	Fourier projection on 1 plane		Preliminary report	(280)
Dibenzylphosphoric acid	3-dimensional Fourier analysis	0.03 A	Determination of dihedral angle in phosphate ester links	(66)

* See footnote 4.

two purines; the purine analogue xanthazole which has three nitrogens next to each other in the five-membered ring; two nucleosides; one nucleotide (partly determined); and a diester of phosphoric acid. By telling us the exact bond distances and angles, and, in some compounds, the positions of the hydrogen atoms, the analyses of the pyrimidines and purines allow distinctions to be made between the various possible tautomeric forms, as well as

allowing the different resonance structures contributing to the tautomers to be calculated. A classic example is Cochran's work on adenine hydrochloride, in which he succeeded in determining the electron density distribution in a Fourier projection with the unusually high accuracy of 0.1 electron /A². He then calculated a contour map showing the difference between the observed distribution of electron density and that to be expected if the diffraction pattern were due only to scattering by the chlorine, carbon, oxygen, and nitrogen atoms. This difference Fourier (Fig. 3) shows striking peaks at the

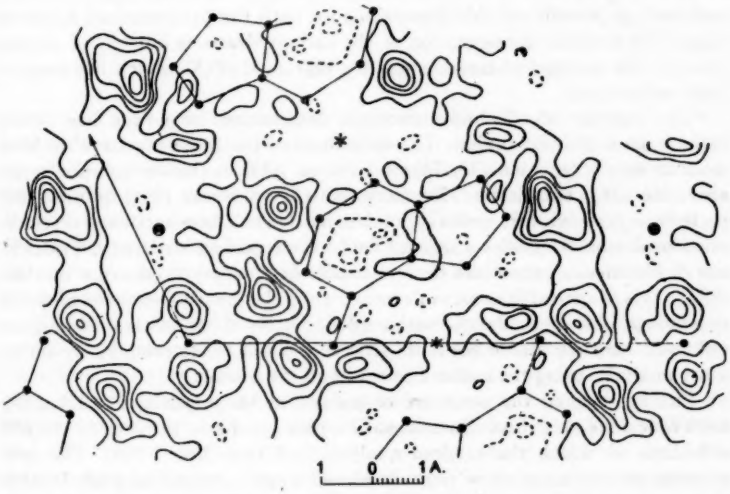


FIG. 3. Difference between the observed electron density of adenine hydrochloride and that calculated for isolated Cl, O, N, and C atoms whose centres are indicated by dots. The large dots indicate chlorines and the two small dots along the horizontal axis represent the oxygens belonging to two water molecules. The peaks in this contour map indicate the hydrogen positions. [Reproduced from Figure 6 in Cochran, W., *Acta Cryst.*, **4**, 87 (1951).]

positions of the hydrogen atoms, which had always been believed to make too weak a contribution to the x-ray scattering to allow them to be located with accuracy. The structural information which emerged from this work later helped Watson & Crick to decide between the various possible ways of base pairing in molecular models of deoxyribonucleic acid.

Another piece of information, which was still lacking in 1953, is the dihedral angle between the planes of the two ester links of the phosphate group. This has now been determined in dibenzylphosphoric acid by Dunitz & Rollett. It is 88°, which is close to the angle assumed by Watson & Crick. Cochran & Woolfson's structure of adenylic acid is also of great interest,

especially in relation to the still unknown structure of ribonucleic acid, but so far only one projection is available. Adenosinetriphosphate has only just been crystallized for the first time (67), permitting one to hope that an accurate x-ray analysis of it will be published by the time this subject is reviewed again.

Carbohydrates, alkaloids, and miscellaneous compounds.—The number of carbohydrate structures known is small: glucosamine (68); glucose (69); sucrose (70, 71); fructose [(72) incomplete structure]; and ribose, determined as part of cytidine (62), adenylic acid (64), and vitamin B_{12} (13). The list is sufficient to provide reliable dimensions for both the pyranose and furanose rings. The absolute configuration of the carbohydrates is known, of course, through the analysis of tartaric acid by Bijvoet *et al.*(5), which has already been referred to.

The number of alkaloid structures determined by x-rays has grown rapidly since the last review. The structures of the following have all been worked out: strychnine (73, 74); colchicine [(75) tentative solution]; isosparteine (76); ephedrine (77); morphine (78); codeine (79); quinine (80, preliminary structure); and cryptopleurine (81). These intricate three-dimensional molecules offer a fruitful field for stereochemical study. Fridrichson & Mathieson's structure determination of *dl*-isocryptopleurine methiodide (81) is of special interest in demonstrating the greatly increased power of crystallographic methods: the authors deliberately chose an alkaloid of quite unknown constitution to see if its structure could be determined by direct x-ray analysis, using the iodine atoms to find the phases.

Pitt has refined the structure of potassium benzylpenicillin (82) using 1680 reflexions in a three-dimensional Fourier synthesis, in place of the 650 reflexions on which the original analysis had been based (83). The new electron density maps show improved bond lengths, increased peak heights and much better resolution. All the original results about the chemical structure of penicillin are confirmed.

In conclusion we should like to draw attention to the structure of an inorganic compound much used by biochemists: sodium dithionite, $Na_2S_2O_4$ (84). Dunitz's structure determination shows the dithionite ion to resemble a builder's trestle (Fig. 4). The S—S bond (2.39 A) is 0.3 A longer than a normal single bond, giving a bond number of only 0.36. The most peculiar feature, however, is the proximity of the two pairs of negatively charged oxygens on the same side of the sulphur atoms. Dunitz gives an interesting analysis of the structure in terms of molecular orbital theory.

Nucleic Acids, Nucleoproteins, and Synthetic Polynucleotides

Deoxyribonucleic acid (DNA).—Watson & Crick's double helix model of DNA (85, 86, 87) has aroused widespread interest and has already been the subject of several reviews (88, 89). Put forward as a tentative structure at first, it has since been confirmed by further x-ray work and by many physicochemical and biophysical techniques. The detailed parameters of the first

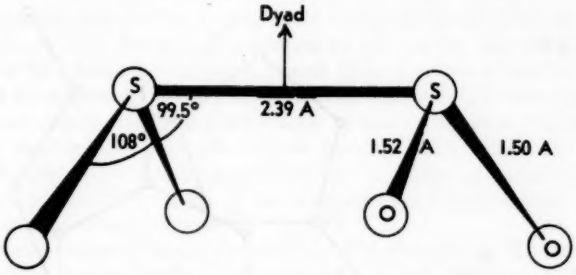


FIG. 4. The structure of the dithionite ion ($S_2O_4^{=}$.)

model (87) have been revised in the light of improved x-ray pictures by Wilkins and his collaborators at King's College, London (90). Figure 5 gives the approximate atomic positions in this revised structure, and shows two successive residues in one of the chains, seen from the direction of the fibre axis. If this drawing is compared with Figure 7 in Watson & Crick's original paper (87), it is seen that the new helix is more tightly wound, all the atoms being nearer to the central axis, and that the phosphate groups are twisted from the original position.

Figure 5 is derived from the x-ray picture known as type B and corresponds to the structure of DNA at humidities above 80 per cent. At lower humidities the fibre shrinks lengthwise and forms a structure of high crystallinity (type A) giving a far more detailed x-ray photograph. Transition between types A and B is reversible. Therefore, if the double helix of Watson & Crick correctly represents type B, a closely related structure should be in agreement with the x-ray pictures of type A. Independent analyses by different workers (91, 92), using entirely different methods of approach, have confirmed that the type A pattern is consistent with a double helix having a pitch of 28.1 A, with 11 residues per turn in each helix. The structure is derived by lengthwise contraction of the Watson-Crick model, making their two chains equidistant and tilting the base pairs by 25° relative to the normal to the fibre axis.

Nucleoprotamine and nucleohistone.—New highly crystalline x-ray pictures of DNA type B, obtained by Wilkins and his collaborators (93), bear a striking resemblance to pictures of oriented, live sperm heads of sepia and loligo (cuttlefish and squid). Reflexions occur in similar positions on the two films, but the intensity distribution is different, especially in the first layer line which is weak in DNA and strong in sperm heads. Wilkins *et al.* (93) suggest that the difference could be explained by postulating that a third chain, consisting of protamine, is wrapped round the double helix of DNA, such that each phosphate in DNA forms a salt link with an arginine of the protamine chain. The detailed features of this model are of course tentative, but perhaps the most significant aspect of the work lies in its proof that the

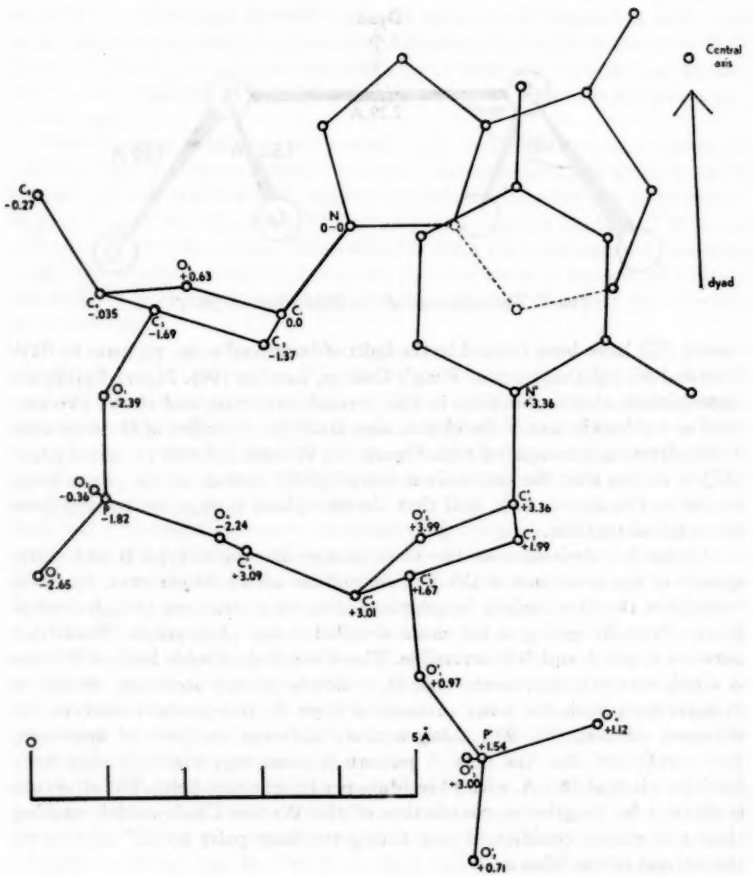


FIG. 5. Two successive residues in one of the chains of the DNA double helix. The numbers indicate heights of the atoms in Ångstrom units above and below the plane of the paper. These coordinates are only approximate and further refinement of the structure is still in progress. (Drawn from data supplied by Dr. M. H. F. Wilkins.)

double helix of DNA exists in the chromosomes of live sperm heads, and that it is not an artefact produced on purification of DNA. Pictures of nucleohistones from various sources have also been taken (94). They also show close similarities to DNA but are of poorer quality than the pictures of nucleoprotamines and, unlike the latter, they show a weak first layer line. A structure has not yet been proposed.

Ribonucleic acid (*RNA*).—The structure analysis of RNA is proving more difficult than that of DNA, because no one has yet succeeded in aligning the RNA chains into a well-oriented fibre. Pictures of partly oriented material have been taken by Rich & Watson (95, 96). The fibres are negatively birefringent, indicating orientation of bases normal to the fibre axis, but the birefringence is much weaker than in DNA. The x-ray pattern resembles that of DNA in its general character, indicating vague similarities in structure, but the spacings of the reflexions are different from DNA. A model has not yet been proposed.

Synthetic polynucleotides.—Just as the structure of the fibrous proteins was finally solved by working on the simpler synthetic polypeptides, so the preparation of synthetic polyribonucleotides by Grunberg, Ortiz & Ochoa (97) has recently diverted the attack on RNA to easier ground. So far x-ray fibre diagrams have been obtained from polyadenylic acid (poly-A); from copolymers of adenylic and uridylic acid (poly-AU), and of all four bases present in RNA (poly-AGUC); and finally from a mixture of polyadenylic and polyuridylic acid (poly-A+poly-U) (98, 99).

Poly-AU and poly-AGUC give x-ray pictures most closely resembling those of natural RNA. Seeing that the bases in these polymer chains are strung together at random, this resemblance excludes specific base pairing as a dominant feature of the structure of RNA. Poly-A gives a highly crystalline x-ray fibre diagram of a type which suggests a helical two-chain structure with eight residues per turn in each helix and with a pitch of 30 A. Watson (99) proposes a model consisting of two helical chains related by a vertical dyad (in contrast to the horizontal dyads operating in DNA), and joined by hydrogen bonds between pairs of adenine. The links run from the NH_2 nitrogen to the free nitrogen in position 7 of the purine ring. This model gives good agreement with the x-ray pictures. There is enough resemblance between the fibre pictures of poly-A and RNA to suggest a fairly close relation between the two structures.

Rich & Davies recently made some most interesting observations when they mixed together solutions of poly-A and poly-U (100). There was an immediate and dramatic increase in viscosity, as Warner (101) had found, and from the viscous solution negatively birefringent fibres could be drawn which gave a strikingly helical x-ray pattern. This pattern is readily interpreted in terms of a double helix in which uracil and adenine are linked together by hydrogen bonds. Each helix has about 10 residues per turn and a pitch which varies between 32 and 36A, depending on humidity. The implications of this discovery are twofold. It shows that ribo- as well as deoxyribonucleotide chains can form double helices linked by hydrogen bonds between specific base pairs and it also proves that two structurally complementary chains can coil rapidly round each other in solution; hence, the mechanism of duplication of DNA proposed by Watson & Crick need not be regarded as an improbable process merely on account of the large amount of uncoiling and coiling of long chain molecules which it would involve.

Synthetic polypeptides and fibrous proteins: alpha structures.—When the structures of biological macromolecules were last discussed in these volumes there was no generally agreed model for the chain configuration in the α-group of fibrous proteins. Just about the time, however, that this discussion was published, the whole field was transformed by the discovery of the α-helix (in 1951) by Pauling, Corey & Branson (102, 103). Since then it has come to be generally accepted that the structures of the synthetic polypeptides and of the fibrous proteins in the α-form are based essentially on this helix, which has been treated in detail in many review articles, among which may be mentioned those of Pauling (104), Perutz (105), Kendrew (106), Crick (107), as well as several papers presented to conferences organized by the Royal Society in 1952 (108) and by the Society for Experimental Biology in 1954 (109). Most of these reviews also cover the other topics discussed in this section. It is unnecessary to do more here than mention that the α-helix is built from a single polypeptide chain and contains 3.6 residues per complete turn of 5.4 A, so that each residue accounts for 1.5 A length of helix, the chain being held in its helical conformation by internal hydrogen bonds. Since 1951 much detailed information about the synthetic polypeptides has been published [e.g. see the series of papers by Bamford, Elliott, and their collaborators (110, 111, 112)], and many studies have been made with the object of confirming that the α-helix is indeed present in them; we shall mention the most important. (*a*) Interpretation of x-ray photographs in terms of helices was greatly facilitated by Cochran, Crick & Vand's publication of a simple but comprehensive theory of diffraction from helical structures (113). (*b*) As a direct experimental test for the structure, Perutz pointed out that the α-helix alone, among the chain configurations which had been proposed, should give a strong 1.5 A meridional reflexion (corresponding to the length of helix occupied by a single residue), and found it to be present in synthetic polypeptides and also hair, porcupine quill, muscle, and other proteins (105, 114, 115). (*c*) The radial distribution function for the x-rays scattered from amorphous material has been calculated by Donohue (116) for the α-helix, as well as for other proposed configurations, and agrees well with that experimentally observed (117); this function is not, however, a very sensitive test of the configuration. (*d*) Measurements of the infrared dichroism of synthetic polypeptides have indicated that the dichroic ratio of the NH stretching frequency is always greater than that of the CO stretching frequency, although in the α-helix these two bonds are virtually parallel and only 12° from the helix axis; for several years the discrepancy was thought to be serious. Theoretical calculations by Fraser & Price (118) have, however, indicated that the transition moment for the CO bond lies at an angle of 20° away from the bond direction, which goes far to explain the discrepancy; recently experimental confirmation of this effect has been obtained [see especially Bailey (119) and Sandeman (120), who compared the crystal structure and crystal dichroism of N, N′-diacetyl-hexamethylene-diamine, which forms triclinic crystals with all the molecules parallel]. (*e*)

More directly, there have been several detailed comparisons between the observed x-ray pattern of synthetic polypeptides and that predicted on the basis of the α-helix (121, 122, 123, 223), and apart from minor difficulties which further work will probably resolve there is now no serious doubt of the presence of the α-helix in the synthetic polypeptides. Other techniques have confirmed that it also exists in solution and can break up or re-form according to the environment [see e.g. Doty, Bradbury & Holtzer (124)]. Elliott & Malcolm (223) found poly-L-alanine to consist mainly of right-handed helices, corresponding to the α_3 structure of Pauling & Corey, which makes it probable that other L-amino-acid polymers also form right-handed helices (see p. 353).

When we turn to the naturally occurring materials in the α-configuration, such as keratin and muscle, the position is not quite so simple. It was pointed out at an early stage that the α-helix as it stood could not explain even some of the rather striking features of the observed diffraction pattern (125); nevertheless, in general, this pattern, though more diffuse, resembled that of the synthetic polypeptides so closely as to leave no real doubt that the α-helix was somehow involved—besides which the diagnostic 1.5 A reflexion was always present. The difficulty was resolved, at least in principle, by a suggestion made independently by Crick (126, 127) and by Pauling & Corey (128) to the effect that the whole helix itself might be twisted into a super-helix or coiled-coil; they showed in general terms that with this modification the observed diffraction pattern could be accounted for. Recently the theory of the coiled-coil has been worked out in somewhat more detail, and compared directly with experiment [Lang (129)]. Thus, although details remain to be settled it appears certain that the α-proteins as well as the α-forms of the synthetic polypeptides have structures based on the α-helix, though in the former case there are in addition higher forms of organization such as the coiled-coil.

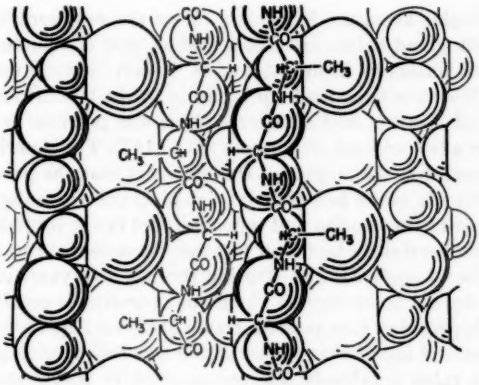
Beta structures.—Ever since the classical studies of Brill, Meyer, Mark, Astbury, and others it has been clear that the β-forms of synthetic polypeptides, keratins, and silks contain chains of amino acid residues in almost completely extended form. During the period under review these ideas have gained some precision, though it is not clear that finality has even now been attained. In the fully extended polypeptide chain CO groups stick out alternately on the two sides of the chain, as do the NH groups, and one might imagine a structure in which parallel chains of this kind are linked together by hydrogen bonds between these groups into an infinite network. Such a fully extended chain would, however, have a repeat distance greater than that observed in any actual structure, and would in any case be impossible for any polypeptide, except polyglycine, owing to steric hindrance between side and main chains.

After a preliminary proposal (130), since abandoned, Pauling & Corey suggested a modified version of the fully extended chain (131), in which the extension is somewhat reduced, but the CO groups (and NH groups) still

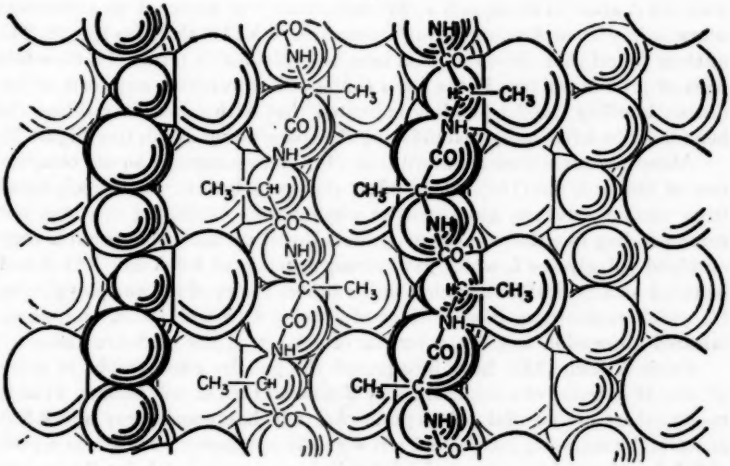
protrude alternately on opposite sides. Neighbouring chains can be joined together to form sheets of two sorts, according to whether neighbours have the same orientation (parallel pleated sheet) or opposite orientation (antiparallel pleated sheet). The directions of CO and NH groups are such that hydrogen bonds can be made between them in either case. In the parallel pleated sheet the repeating unit is 6.50 A, and this is the structure proposed for β-keratin; in the antiparallel sheet the unit is 7.0 A, and this is proposed for silk (132). There seems little doubt that structures of this general type are in fact present in these substances, but so far very little work of a quantitative kind has been done to compare observed and calculated data, at any rate in the synthetic polypeptides or in β-keratin. Marsh, Corey & Pauling (133) believe that the x-ray pattern of β-polyalanine can best be explained in terms of antiparallel sheets, while Brown & Trotter (134) consider that no final decision can yet be reached as to which type of sheet is present.

Much more detailed work has been done on silk fibroin. It has been shown (135) that three main types of silk fibre can be distinguished by their diffraction patterns: that of *Bombyx mori*, with axial repeat distance of 9.3 A, that of *Antherea* spp. (otherwise known as Tussah silk), with repeat of 10.0 A; and finally that of *Anaphe* spp., with repeat of 10.6 A. Complete amino acid data are not available except for *Bombyx* silk, which has the approximate composition $Gly_3Ala_2X_3$; that of Tussah silk is more nearly $Gly_2Ala_3X_3$ (136). Detailed studies of *Bombyx* silk have recently been made by Warwicker (137) and by Marsh, Corey & Pauling (138). In each the conclusion was that the structure is made up of antiparallel pleated sheets, whose chains consist for the most part of alternate glycine and alanine residues, so that the methyl groups of the alanine stick out entirely on one side of the sheet, while the other side of the sheet has only hydrogen atoms in the corresponding positions; thus pairs of sheets are separated alternately by distances of 3.5 to 3.7 and 5.6 to 5.7 A. Warwicker arranged his sheets in an orthorhombic cell of space group $P22_12_1$. This arrangement has been criticised by Marsh *et al.* (139) because it does not satisfactorily predict the intensities of two important reflexions (120 and 020). Instead, the latter authors prefer a monoclinic cell, space group $P2_1$, with $\beta = 90°$ (see Figure 6a). In neither case can the residues larger than alanine (they make up about 18 per cent of the protein) be accommodated between the sheets. It is suggested that they are inserted at random in the structure, causing occasional distortion of the sheets which would account for some of the diffuse reflexions always observed.

Tussah silk has a diffraction pattern resembling that of *Bombyx* silk, but not identical with it. It is, in point of fact, virtually isomorphous with β-polyalanine (140). In a detailed study. Marsh, Pauling & Corey (133) conclude that, as before, antiparallel pleated sheets are present, but that the mode of packing is different, all pairs of sheets being separated by 5.3 A (see Figure 6b). This is a consequence of the fact that in Tussah silk the



(a)



(b)

FIG. 6a and b. Structure of (a) *Bombyx mori* and (b) Tussah silk. [Reproduced from (a) Figure 11 of Marsh, Corey & Pauling, *Biochim. et Biophys. Acta*, **16**, 18 (1955) (Elsevier Publishing Company, Amsterdam); (b) Figure 3b of Marsh, Corey & Pauling, *Acta Cryst.*, **8**, 713 (1955)].

percentage of alanine is higher, and that of glycine lower, than in *Bombyx*, so that sequences of the type —G—X—G—X— can no longer make up a significant proportion of the whole, with the result that the methyl groups of alanine must stick out indifferently on the two sides of any one sheet, which thus become structurally equivalent.

A considerable literature has grown up on the configuration of silk protein in the silk gland before extrusion, and on that of soluble silk, made by dissolving silk fibroin in lithium bromide. X-ray, electron-microscope, and infrared studies have been made, principally in the laboratories of Bamford and Elliott, of Kratky, and of Mercer. It is not possible here to do more than mention a few selected references (141 to 147). The experimental results are complicated, but the essence of the matter is that the protein present in solution in the silk gland is a globular one of unknown configuration; while if silk is brought into solution and reprecipitated in the form of a film, it will under suitable conditions be found to have the α-configuration.

Polyproline: polyglycine II.—During the last two years it has emerged that a hitherto unknown type of chain configuration is possible for certain synthetic polypeptides. Cowan & McGavin (148) obtained fibre photographs of polyproline and found that the structure was helical with an axial repeat of 9.36 A—a value previously not encountered in polypeptides. Measurements of density and cell dimensions indicated that the polypeptide chains were 6.6 A apart in hexagonal array; each chain was centered on a threefold screw axis, with prolines repeating at intervals of 3.12 A along its length. The authors found that the data could best be explained in terms of a threefold helix of proline residues in the *trans* configuration, without any form of hydrogen bonding. Model building indicated that with L-proline residues the helix must be left-handed, establishing the space-group as $P3_2$ (see Figure 7).

About the same time Bamford *et al.* (149) reinvestigated an old observation of Meyer & Go (150), to the effect that polyglycine, when precipitated from certain solvents, gave powder photographs exhibiting spacings not corresponding to either α- or β-structures. Under certain circumstances they obtained polyglycine I, which gave strong spacings of 4.4 A and 3.45 A and behaved as a typical β-structure; under others the product was polyglycine II, which gave strong powder rings of spacing 4.15 and 3.1 A, and whose infrared absorption was characteristic of neither α- nor of β-structures.

Crick & Rich (151) have interpreted the powder photographs of polyglycine II in terms of a structure very similar to that of polyproline. That is to say, there are parallel polypeptide chains in hexagonal array and 4.8 A apart each possessing a threefold screw axis of symmetry with a true repeat of 9.3 A and a residue repeat of 3.1 A; the space group is $P3_1$ (or $P3_2$—since glycine residues are optically inactive the two are indistinguishable). In this structure, however, each residue is hydrogen-bonded to a neighbour in the next chain, and the hydrogen-bond direction is roughly perpendicular to the chain axes. Thus the whole structure is held together by an infinite network of hydrogen bonds. Reasonable qualitative agreement between calculated and observed intensities was obtained. It is remarkable that after so many years in which virtually all known polypeptide fibre structures fell into either the α-, the β-, or the collagen classes, two representatives of a totally new one should appear almost simultaneously.

Collagen.—Besides the general reviews mentioned above, there have

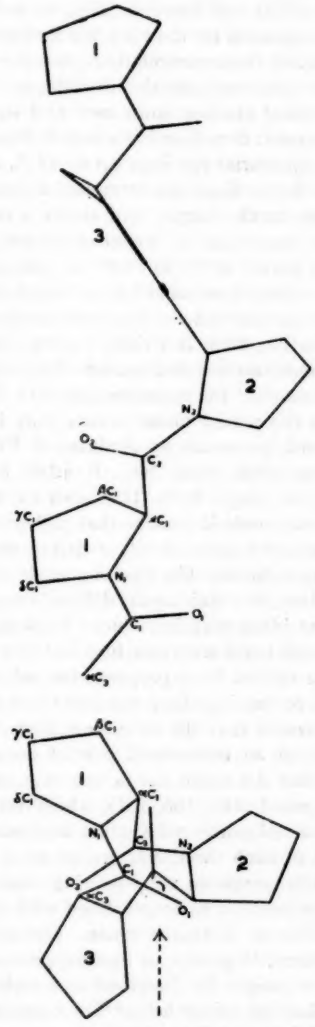


FIG. 7. Structure of polyproline seen, below, along the direction of the threefold axis and, above, perpendicular to this axis and along the direction of the arrow. [Reproduced from Figure 2 of Cowan & McGavin, *Nature*, 176, 501 (1955).]

been several dealing specifically with collagen, among which may be mentioned those by Bear (152) and Randall (153), as well as the published report of a conference organized by the Faraday Society (154) and a book by Gustavsen (155). None of these compilations, however, contains an account of recent work on the chain configuration of collagen.

Apart from theoretical studies, some new and significant experimental facts have been discovered: thus North, Cowan & Randall (156) have found a series of low-angle equatorial spacings up to 49 A, and the same authors have shown that if collagen fibres are stretched as much as 8 per cent, the x-ray pattern becomes much sharper and shows a number of new details which have been very important in recent structural studies (157, 158).

At the end of the period under review the question of the polypeptide chain configuration in collagen seems at last to have been more or less settled, as a result of studies carried out by four independent groups of workers: Ramachandran & Kartha; Rich & Crick; Cowan, North & Randall; and Bear. This successful outcome has terminated a long series of proposals, none of which was found to satisfy the experimental data. Among the unsuccessful ones made during the period under review may be mentioned, for the sake of historical record, proposals by Ambrose & Elliott (sheet structure, 159), Pauling & Corey (triple helix, 160), Randall, Fraser & North (sheet structure, 161), Huggins (single helix, 162), and Crick (double helix, 163).

The recent work has made it certain that collagen in fact consists of a triple helix of polypeptide chains, slowly twisting round one another and linked across by hydrogen bonds. The idea of a triple helix was first proposed by Pauling & Corey (160), but their model differed completely from the more recent ones. The newer ideas were introduced by Ramachandran & Kartha (164) who at first postulated a structure in which the configuration of each chain was very similar to that later proposed for polyproline, that is to say with a repeat of 9.5 A containing three residues related by a threefold screw axis. This model demanded that the prominent 2.86 A meridional reflexion should in fact consist of an unresolved pair of nonmeridional reflexions; later work indicated that this could not be the case, and a revised model of the structure was proposed (165, 166, 167), which differed from the first in that the chains now formed gentle coiled-coils, repeating after about 28.6 A. In this structure, out of each three NH groups on a single chain two are hydrogen-bonded to CO groups on neighbouring chains; the CO group left unbonded in each triad forms a hydrogen bond with the hydroxyl group of a hydroxyproline residue on a nearby chain. The sequence of residues is —G—R—P— (G, glycine; P, proline or hydroxyproline; R, other residues). The chain configuration might be described as a right-handed major helix superposed on a left-handed minor helix.[8] The orientation of the hydrogen bonds agrees well with infrared data (168).

[8] The terms "major" and "minor helix" have been introduced by Crick (127) in his theory of diffraction from coiled coils. The "minor helix" defines the turns of the residues of one individual chain around its own axis; e.g. the pitch of 9.5 A corresponding to the repeat pattern of three residues in the individual collagen chain

Substantial modifications of the same basic ideas were proposed by Rich & Crick (169), who criticised the structure of Ramachandran & Kartha on stereochemical grounds, and also because the sequence —Gly. Pro. Hypro—, shown chemically to be common in collagen, would be inadmissible in this model. They proposed two related models which they have called collagen I and collagen II, both of which accommodate the above-mentioned sequence, and both of which are also closely related to the structure of polyglycine II. The principal difference between the two models is that collagen I has systematic hydrogen-bonding between the CO groups of one chain and the OH groups of the hydroxyprolines of a neighbouring chain, while collagen II, which is shown in Figure 8, has not.

At about this time Cowan, McGavin & North (170) also presented some discussion of triple chain helical models, based on the structure of polyproline, without, however, proposing a precise structure. Bear (171) made an independent deduction of the three-chain model by a different method and showed that Rich & Crick's collagen II best satisfied the observed optical transform. Meanwhile Ramachandran (172), too, had been thinking about the two possible variants of the original model, and now prefers the model described as collagen II, in which the sequence —G—R—R′— is permissible, without special limitations as to the nature of R and R′. This is how the problem stands at the present time.

Finally, it is of interest to note that various studies, in solution, of soluble collagen (tropocollagen) and of gelatin, by the techniques of optical rotation, infrared spectroscopy, and light-scattering, have also indicated that native collagen has a helical structure (173, 174), and have shown that the results are best fitted by a triple helix of diameter 13.6 A and over-all length 3,000 A with a molecular weight about 345,000 (175).

Theoretical studies of polypeptide chain configurations.—The intensive studies of fibrous proteins of the past few years have led to the description of a number of other possible chain configurations which, while satisfying all or most of the structural criteria of bond length and bond angle, have been proposed as constituents of real substances in a manner at most tentative. Thus Pauling & Corey (102) described a γ-helix of much larger diameter than the α-helix; while Low & Baybutt suggested the π-helix, intermediate between the two, as a possible constituent of α-structures (176, 177). A large number of configurations with *cis* amide groups has been described by Pauling & Corey (178, 179), as well as two near relatives of the pleated sheets which could accommodate alternate L- and D-residues (180). A valuable study of the relative stabilities of various helical models has been made by Donohue (181); he showed that of all the models so far put forward the α-helix is the most stable, with the π-helix not far behind. Another theoretical study by Low & Grenville-Wells (177) developed general methods for

(Fig. 8b). The "major helix" defines the turns of the entire chain around an outside axis, e.g. the pitch of 3×28.6 A of each of the three chains winding around the common axis in Fig. 8a.

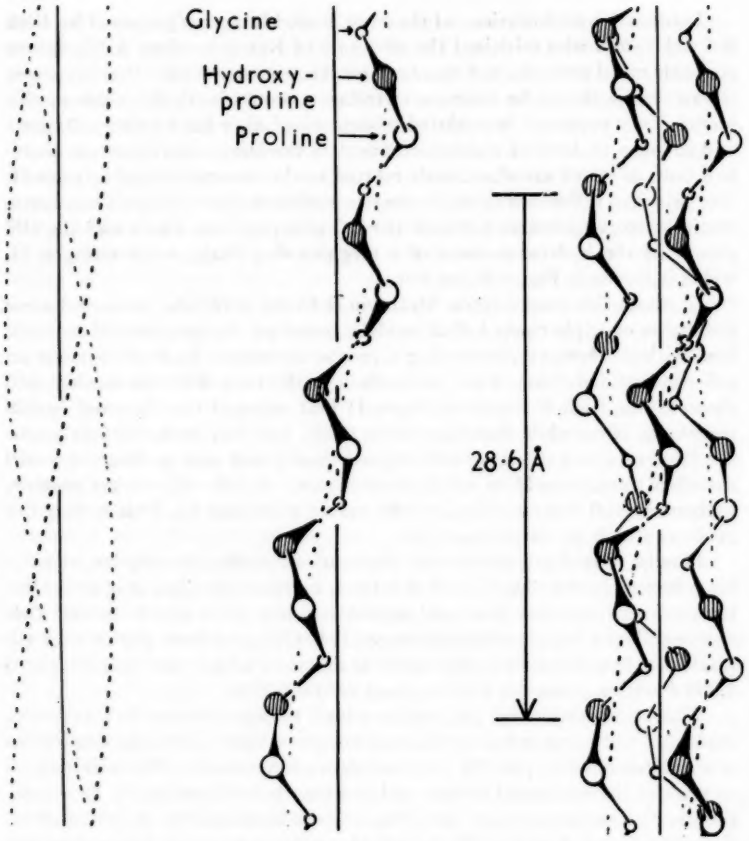


FIG. 8. Structure of collagen: diagrammatic representation of the triple chain structure, in which each residue is shown as a circle. The broken lines represent the screw axes of the individual chains. (a) Interlacing arrangement of three chains. (b) A single chain, showing the helical coiling about the chain axis. (c) The complete structure. (Drawing supplied by Dr. F. H. C. Crick.)

calculating the co-ordinates of atoms in helical structures. Finally, Lindley (182) has investigated ways in which the direction and sense (left- or right-handed) of the α-helix might be modified.[6]

Evidence for large units of structure in fibrous proteins.—For many years

[6] Bamford, Elliott & Hanby have just published a monograph on synthetic polypeptides in which many of the problems reviewed here are discussed in detail (285).

evidence has been accumulating that in fibrous proteins the folded polypeptide chain does not represent the highest level of organization present, but that the chains are organized into subunits, large compared with themselves, and that the visible fibrils are made up of aggregates of these subunits. This evidence has been both chemical and physical; thus disaggregation of the fibre has led to the production of globular soluble proteins of a more or less homogeneous kind in solution, while electron-microscope and x-ray studies have indicated that large units are present in the intact structure. Recent appreciation of the importance of the helix as a structural principle of wide significance, both at the level of the chain and at higher levels in certain instances (e.g. tobacco mosaic virus, see p. 360), has given rise to speculations that the subunits are generally arranged in helical aggregates [see, for example, Pauling (183)]. Only in the case of tobacco mosaic virus, however, have hypotheses of this kind gone beyond the stage of speculation to a model which can be tested against experiment. During the period under review new experimental material has appeared at so high a rate that we can do no more here than mention its existence. In collagen, for example, the low-angle x-ray pattern and the fine-structure bands visible in the electron microscope have been studied intensively by Schmitt, Bear, Gross, Highberger and their associates, in parallel with chemical studies which have culminated in the isolation of a homogeneous soluble protein called tropocollagen; this appears to be the basic subunit of the structure, and tentative hypotheses as to its mode of aggregation have been put forward [see, for example, (152, 184 to 188)]. Analogous studies have been made by Randall and his collaborators (158, 189) and by Tomlin & Worthington (190). The low-angle diffraction pattern of keratin fibres (especially porcupine quill and feather) has been the subject of several attempts at interpretation, by Pauling & Corey (191); Bear & Rugo (192), Krimm & Schor (193), and others. Actin has been studied by Cohen & Hanson (194) and Selby & Bear (195), myosin by Szent-Györgyi (196, 197) and Tsao (198), and from the x-ray point of view by Bear & Selby (199).

Globular Proteins

Crystals of many globular proteins have now been studied by x-rays, but few of the studies have gone beyond the preliminary stage of working out the unit cell dimensions and space group, and of calculating one or two Patterson projections. For a table of such x-ray data on proteins see Kendrew (200). In this review we should like to report on attempts at detailed structure analysis in progress on four proteins: insulin, haemoglobin, myoglobin, and ribonuclease. Apart from Lindley & Rollett's instructive model building of insulin, none of the analyses has, as yet, yielded much information about the structure of the protein molecules; however, the methods of approach have improved enormously both in power and exactitude, and for the first time electron density maps are being obtained which can be proved beyond reasonable doubt. These maps have led to the sobering realization

that possibly no solution of the problem may be expected on the basis of any one simple structural hypothesis and that nothing short of direct x-ray analysis in three dimensions is likely to lead to ultimate success. It seems that such an analysis is possible, at least with a protein of small molecular weight, but it will be a formidable task.

Insulin.—This protein has been studied in two forms: in a rhombohedral one, as the zinc salt, by D. Crowfoot-Hodgkin (201); and in an orthorhombic one, as the sulphate, by B. W. Low (202, 203). Three-dimensional Patterson syntheses have been calculated for both forms (201, 204), but neither of these has been published in full. The Patterson of the rhombohedral form does not exhibit any features which readily lend themselves to interpretation, but that of the air-dried orthorhombic one shows rod-like concentrations of vector density which Low has tentatively interpreted in terms of parallel polypeptide chains 44 A long, spaced 10 to 12 A apart. The resolution of these Pattersons is insufficient to determine the repeat of the pattern along the chains, which makes it difficult to guess their configuration; nor can one tell, without knowing the absolute height of the vector peaks, how large a fraction of the insulin molecule is represented by parallel rods [see Crick (205, 206)]; the results are therefore uncertain.

By elucidating the chemical constitution of insulin (207), Sanger has presented crystallographers with a challenge to try to find a uniquely stable configuration of the molecule. Lindley & Rollett, who were the first to attempt this task since the publication of Sanger's final paper, assumed the α-helix configuration as the main basis of their model (208). Now Sanger's results show a cystine bridge linking together the sixth and the eleventh residues of chain A, a feature which cannot be fitted into a single, straight α-helix. After many trials Lindley & Rollett found the most satisfying solution to be a left-handed helix from residue A1 to A9 which changes its sense at the disulphide bridge and continues as a right-handed helix from A9 to A21. The transitional region where the helix reverses its sense is stabilized by a number of hydrogen bonds between the two portions of the chain, leaving only the NH group of residue 12 completely unbonded. Chain B has the form of a right-handed α-helix throughout. The chains A and B are linked together by the two known disulphide bridges, by a salt bridge between the arginine B22 and the terminal carboxyl group of chain A, and by van der Waals attraction between four leucines. Two such insulin molecules are then joined together by a zinc atom co-ordinated to two histidine and two glutamic acid side chains. Lindley & Rollett's model appears to be stereochemically satisfactory and to pack nicely into both the rhombohedral and orthorhombic forms. A detailed analysis of the rhombohedral crystal structure in terms of the model, such as would be required to prove it, has not yet been possible.

Linderstrøm-Lang & Schellmann have proposed a rather similar model (209) consisting of a left-handed helix for chain A and a right-handed one for chain B, with the awkward region at the cystine bridge A6–A11 being left

loose. The ends of the two chains, beyond the two cystine bridges, are left unfolded. This model is not as precise as Lindley & Rollett's and was built in order to explain the deuterium exchange properties of insulin in solution, rather than to solve its crystal structure.

Both Lindley & Rollett and Linderstrøm-Lang quote Arndt & Riley's experiments on the radial X-ray scattering curve of insulin (117) in support of their assumption that insulin consists of a mixture of right-handed and left-handed helices. In the reviewers' opinion such scattering curves are useful as qualitative evidence of the presence of α-helices or similar structures, but they are not sensitive enough to allow a distinction to be made between left-handed and right-handed α-helices. Arndt & Riley's conclusion that the left-handed (α_1) configuration is predominant in globular proteins has now been rendered improbable by Yang & Doty's measurements of optical rotatory dispersion (210); it is in fact the right-handed (α_2) helix which predominates, just as it does in synthetic polypeptides (223). Yang & Doty's results are also the more satisfactory from the purely stereochemical point of view. A chain consisting of L-residues would be expected to be less stable as a left-handed helix than as a right-handed one, because the former involves a closer approach between the carbonyl oxygen and the β-carbon atom.

Haemoglobin.—When Pauling & Corey first noted the correspondence between the three-dimensional vector structure of haemoglobin and the vector distribution to be expected from the α-helix (211), and Perutz subsequently found a weak, but detectable, 1.5 A reflexion in haemoglobin (114), Perutz believed that a solution of the haemoglobin structure in terms of parallel α-helices might be found by trial. This led to an attempt by Bragg, Howells & Perutz to calculate the electron density distribution viewed along the chain direction of the molecule (212). A Fourier projection was indeed obtained, but its validity rested on the assumption that the interpretation of the vector structure in terms of parallel α-helices was correct. When the absolute heights of the electron density peaks were measured, they were found to amount to only one third to one half of the heights to be expected on the basis of the initial assumptions, which showed that the model was oversimplified. Crick arrived at the same conclusion by a different argument (205, 206) and also pointed out that α-helices are likely to pack together at an angle of about 20°, which speaks against their being parallel in haemoglobin (127).

The failure of this attempt at analysis by trial pointed to the need for a different approach, and one that was free from any preconceived notion about the structure of the molecule. In practice this meant that the phases of the diffracted rays would have to be determined by direct methods. In a series of papers, Bragg, Perutz and their collaborators have now described four different direct methods of phase determination. (These methods apply only to a projection of the crystal structure in which the phase angles are restricted to 0 or π.)

(*a*). Crystals of horse methaemoglobin consist, to more than half their

volume, of liquid of crystallization. In consequence electrolytes can diffuse into the interstices between the haemoglobin molecules. The ensuing changes in the density of the interstitial liquid cause alterations in the intensities of the low order spectra, which may be used for phase determination (213, 214).

(*b*). Haemoglobin crystals can be made to swell and shrink within wide limits, a property which can be exploited to find the nodes and loops in the diffraction pattern of a single haemoglobin molecule. This method gives relations between phases, but not their absolute sign (213, 214).

(*c*). The addition compound, imidazole haemoglobin, crystallizes in a form derived from normal haemoglobin by the inversion of alternate molecular layers. Comparison of the diffraction pattern of the two forms allows many phase relations to be derived (215).

(*d*). Native horse haemoglobin contains four free sulphydryl groups of cysteine which combine with up to four silver ions, or with two moles of *p*-chloromercuribenzoate, or with other mercurials (216). On crystallization, these heavy metal compounds are found to be isomorphous with normal haemoglobin. The presence of the heavy metals produces changes in the intensity of the diffracted rays sufficiently marked to allow their phases to be determined (217). This method proved to be the most powerful of all the ones that were tried; out of a total of 94 reflexions covered by the x-ray photograph, the phases of 87 were fixed by either the mercury or the silver compound or by both, leaving only seven reflexions in doubt. When the phases and the phase relations established by the four independent methods were compared, not a single discrepancy was found. This remarkable consistency gave Bragg & Perutz confidence that any pictures of the haemoglobin molecule derived from these measurements would be correct.

The pictures take the form of contour maps in projection on the *b* plane of the monoclinic crystal (218). Two kinds of map were calculated. In the first kind the changes in amplitude produced when salt solution replaced water as the suspension medium were used as coefficients of the Fourier terms. The resultant map shows the projected thickness of hydrated protein into which salt cannot penetrate. Figure 9 is a picture of the hydrated haemoglobin molecule, derived from that map by separating the contours due to the overlapping of molecules at the corners and centre of the unit cell. (The procedure used for separating the contours was plausible, but not rigorous.) The molecule is seen to correspond closely to the ellipsoid 55 by 55 by 70 A derived earlier by Bragg, Howells & Perutz (219). The most interesting feature is a depression at the centre which apparently corresponds to a dimple located either on one side or on both sides of the molecule. The resolution of the picture is low, because only the lowest order reflexions are affected by changes in the salt concentration of the crystal.

The second map represents the electron density distribution in a single row of haemoglobin molecules suspended in salt-free water (Fig. 10). The molecules are centred on axes of dyad symmetry, one at the centre of the

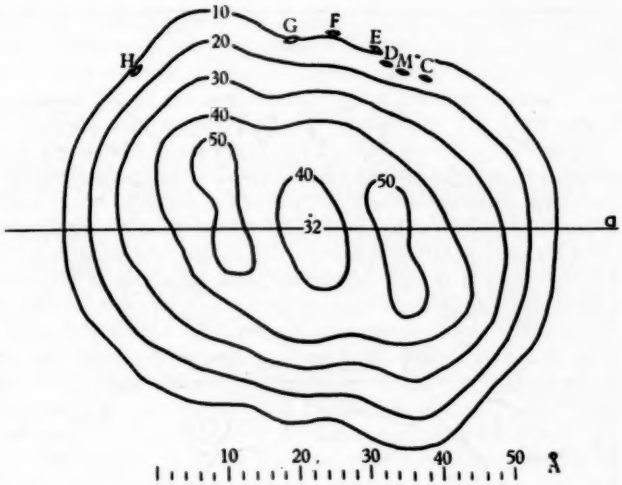


FIG. 9. Tentative contour map of the external form of a single haemoglobin molecule. The numbers represent thickness in Ångstrom units; the letters refer to points of contact between neighbouring molecules at different stages of swelling and shrinkage of the crystal. [Reproduced from Figure 2 of Bragg & Perutz, *Proc. Roy. Soc.* (*London*), [A] **225**, 320 (1954.)]

picture and two others halfway down each side. In space, the latter lie 31.6 A above the molecule at the centre; in projection, the molecules overlap, giving the impression of a continuous row of matter. The external boundary of the molecules lies near the one-electron contour and is seen to be more irregular than Figure 9 indicates. The internal structure exhibits a striking system of peaks and depressions which has so far defied interpretation. The depression near the centre of the molecule appears again, but is now seen to be divided into two depressions on either side of the dyad axis. The lines XX and YY mark two possible cleavage planes where the two halves of the molecule might be joined. The map contains no features which could be identified as iron atoms, haem groups, or polypeptide chains. One reason is lack of resolution; this is so low that single atoms are smeared out to a diameter of 9 A. Another reason is the great thickness of matter, corresponding to about 30 atoms, which is projected onto one plane, thus obscuring the individual parts of the molecule by superposition. Green & North have now partly worked out two more Fourier maps of the molecule, projected in two directions normal to each other and normal to the direction of the first projection, but neither of them is interpretable (220, 221). The only chemical feature determined by the maps is the location of the sulphydryl groups; these are arranged in pairs 30 A apart and about 5 A from the surface of

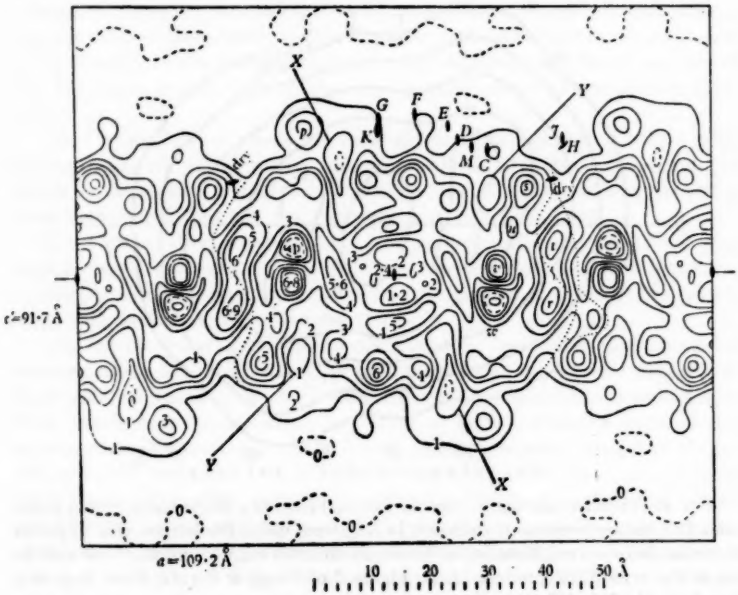


FIG. 10. Projection of the electron density distribution in a single row of haemoglobin molecules, seen at a resolution of about 4 A. [Reproduced from Figure 4 of Bragg & Perutz, *Proc. Roy. Soc.* (*London*), [A] **225**, 320 (1954).]

the molecule. It looks as though only a three-dimensional analysis, giving the electron density distribution in a series of sections through the molecule, will finally reveal its structure.

An interesting piece of information has recently emerged from measurements of electron spin resonance of horse methaemoglobin carried out by Ingram, Gibson & Perutz (222). These give the angular orientation of the four haem groups with great accuracy. There are seen to be two pairs of haem groups in slightly different orientation, the members of each pair being related by the axis of dyad symmetry. Unfortunately the measurements tell us nothing as yet about the actual positions which the haems occupy in the haemoglobin molecule.

Myoglobin.—Early stages in the study of crystalline myoglobin were reported in the last article in this series (1). Progress since then has in the main been a consequence of (*a*) the discovery of many polymorphous forms of myoglobin crystal, and (*b*) the application of the method of isomorphous replacement. All the early work on myoglobin was done with crystals of horse myoglobin, which forms monoclinic (224) or pseudo-orthorhombic (225) crystals. More recently Kendrew & Parrish (226) have obtained upwards of

a dozen new crystal forms of the protein, either by extracting it from the muscles of different species of animals, or by varying the conditions of crystallization from a single species. The most useful classes of animal are the whales, the seals, and the penguins; all these are diving animals and their muscles contain very large amounts of myoglobin. The same crystal form can often be derived from several different species; thus, crystals of the so-called Type A (normally obtained from sperm whale) have been obtained from five different species of whale, the diffraction patterns being only very slightly different in each case, to an extent corresponding to changes in one or two amino-acid side chains. The distribution of crystal forms among species generally (though not always) corresponds to the zoological classification; there is also a correspondence with immunological tests of cross reactions between different myoglobins and their antisera (226).

Some of the crystal forms have been studied in detail by methods based mainly on an examination of Patterson projections and three-dimensional syntheses; for example Tyyes A and B (sperm whale) (227) and Type F (fin-back whale) (228). The Patterson projections of these crystals suggest that at least a proportion of the polypeptide chains in the myoglobin molecule are more or less straight and parallel, though a coiled-coil arrangement of the type discussed in connection with α-keratin (p. 343) would not be excluded. A three-dimensional Patterson synthesis of Type F crystals (Bluhm & Kendrew, in preparation) supports this interpretation. Evidence has also been presented to the effect that the over-all, or packing, shape of the molecule is approximately 40 by 35 by 25 A, the first dimension being parallel to the predominant chain direction. A molecule of this shape packs convincingly into all the crystal lattices so far investigated.

An important question is the position and orientation of the haem group in each molecule. By crystallizing derivatives of myoglobin in which large substituent groups such as isocyanides, imidazoles, and nitroso-compounds are co-ordinated with the iron atom of the haem group, and measuring the unit cell dimensions, Kendrew & Parrish (229) have concluded that the haem group is probably on the surface, since even substituents containing two benzene rings can be attached without appreciably altering the cell dimensions. This conclusion is contrary to that of Lein & Pauling (230), who studied the equilibrium kinetics of combination of isocyanides with the haem group. Information about the orientation of the haem group can be obtained by measurements of optical dichroism of myoglobin crystals in the visible region, or by the technique of electron spin resonance (231). Measurements by these methods give concordant results, and it has recently been shown that in four different crystal forms of myoglobin the angle between the plane of the haem group and the predominant polypeptide chain direction (as deduced from Patterson projections) has a value between 40 and 50° (232).

The application to myoglobin (233, 234) of the principle of isomorphous replacement required the development of new methods, because, unlike

haemoglobin, myoglobin contains no free sulphydryl groups, so that the usual SH group reagents do not combine with it. A survey of ions containing heavy metals showed that in some cases there was a stoichiometric attachment of the ion to some site or sites within the crystal lattice, though the fact that different crystal forms of the protein often behave differently with respect to the same ion indicates that the combination is sometimes interstitial rather than truly chemical. The evidence for attachment was generally crystallographic only; that is to say, distinct changes in the diffracted intensities were observed, without the cell dimensions altering, and these changes were found by calculation to correspond to attachment of a heavy atom at a single site in the molecule. It was found possible to prepare crystals of Type A myoglobin containing mercuri-iodide (HgI_4^{--}), silver (Ag^+), gold (Au^{+++}), mercury-benzenesulphonate ($HO_3S \cdot C_6H_4 \cdot Hg^+$), and others. By correlating the data from all these derivatives the signs of nearly all reflexions in the (010) zone were determined, and in most cases cross-checked from several derivatives, out to a spacing of 4 A. It was then possible to compute a Fourier projection along the *b* axis, and this is illustrated in Figure 11. As in haemoglobin, the degree of overlapping consequent on the great depth of the unit cell through which the projection is made is such that the projection, though almost certainly a correct representation of the projected electron density, is virtually uninterpretable. Not only is it impossible to distinguish internal features of the molecule, but one cannot be certain where one molecule begins and another one ends. The latter point can, however, to some extent be cleared up by the computation of a low-resolution

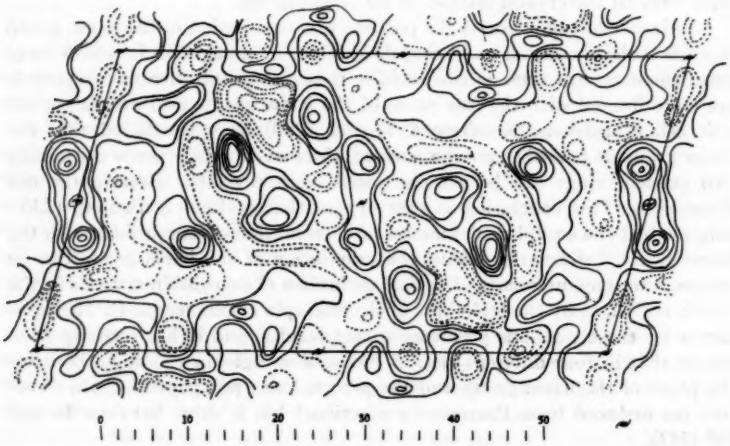


FIG. 11. Projection of the electron density distribution in the unit cell of sperm whale myoglobin, seen at a resolution of about 4 A.

Fourier projection using as terms the rate of change of diffracted amplitude with the density of the salt solution contained in the crystal. Such a projection outlines the regions into which salt does not penetrate, i.e. the regions occupied by protein molecules. It has already been pointed out that the method of isomorphous replacement, in its application to proteins, can only come to fruition when it can be extended to three dimensions. The evaluation of the phase angles of reflexions of general phase, required for a three-dimensional synthesis, involves the preparation of at least two, and preferably three or four, different isomorphous derivatives each having its "heavy atom" at a different site in the unit cell. This condition has now been satisfied in myoglobin, though the next step has yet to be taken.

Other two-dimensional studies of myoglobin include the approximate location of the haem group by labelling with heavy-atom-containing ligands such as *p*-iodo-nitrosobenzene which is known to combine specifically with the iron atom of the haem group; and also the resolution of the mercuri-iodide group into its constituent atoms by examining the mercuri-iodide Type A complex at high resolution.

Ribonuclease.—This protein is being studied in detail in two separate laboratories—that of Bernal and Carlisle in London and that of Harker in New York. Carlisle & Scouloudi (235) have devoted their attention to the monoclinic form of ribonuclease with space group $P2_1$, and two molecules per cell, denoted ribonuclease II by King *et al.* (236). In a study of the two-dimensional Patterson projections of this form they postulate a model consisting of five parallel polypeptide chains parallel to *c*, while a later paper (237) describes (without illustrating) the three-dimensional Patterson function of the crystal. In recent unpublished experiments Carlisle has obtained changes in the diffracted intensities from this crystal by diffusing organic mercurials into it; whether this amounts to an isomorphous replacement remains to be seen.

The New York group of workers has characterized, in all, seven different crystal forms of ribonuclease (236). Detailed work has been done on Type II; for example its three-dimensional Patterson function has been computed (238), and no evidence was found for the existence of parallel polypeptide chains in the *c* direction, as had been postulated by Carlisle & Scouloudi (235). It has also been shown that unless special precautions are taken in mounting crystals for x-ray work there may be rapid fluctuations in the cell dimensions (due to atmospheric temperature variations); these are slight, but sufficient to cause substantial errors in the intensity measurements (239). In unpublished experiments it has been found possible to apply the isomorphous replacement method to this form, by diffusing into the crystal organic dyes containing uranium atoms; stoichiometric combination is obtained, and substantial changes are observed in the diffraction pattern, from which a tentative Fourier projection has been calculated (283). Some study has also been made of ribonuclease VI, a face-centred monoclinic form which is a complex with the dye iodophenol blue; its Patterson projections show

that it is closely related to II (240). More recently the main effort has been devoted to ribonuclease I, an orthorhombic form ($P2_12_12_1$, 4 molecules/cell); isomorphous replacement has been achieved in this form too by diffusing uranium-containing dyes into the crystal (unpublished experiments).

The Viruses

Tobacco mosaic virus.—Earlier studies with the electron microscope (241) and by x-rays (242) had shown that this virus is a rod-shaped object, of length about 3000 A, mean diameter 150 A, and molecular weight 40×10^6 to 50×10^6; it consists of 94 per cent protein and 6 per cent ribonucleic acid (RNA). X-ray results had indicated that in the paracrystal of TMV, in which the molecules are parallel, there was an axial repeat of 68 A; the fact that this is very much less than the length of the particle suggested that the latter must consist of subunits, and some tentative suggestions were made as to their disposition. Since the war the techniques of electron microscopy have vastly improved and much more detailed photographs of TMV have been obtained [see for example Williams (243)].

By the use of carboxypeptidase Harris & Knight (244) showed that the TMV particle contains over 2000 end carboxyl groups all of which are threonine [more recent work by Niu & Fraenkel-Conrat (245) has shown that the C-terminal sequence is in fact -Pro.Ala.Thr]. These results suggest that the TMV particle may contain over 2000 identical amino-acid chains and hence support the idea that it may consist of a large number of identical subunits. A corresponding number of identical terminations has been found at the other end of the chains (246 to 248). Other evidence leads to similar conclusions. Thus several laboratories reported the presence, in plants infected with TMV, of an abnormal protein of low molecular weight associated in some way with the virus (249 to 253), immunochemically related to it and of virtually identical amino-acid composition (254). This protein had the remarkable property of polymerizing to a nucleic-acid free polymer in the form of rods similar to those of TMV itself. It was found possible by several methods to degrade TMV into a mixture of RNA and globular protein of low molecular weight (255). This protein could be separated in a pure and homogeneous form and could be polymerized to rod-like RNA-free structures like those obtained from the naturally-occurring protein just mentioned, or, by adding RNA, to particles very similar to those of the original virus. These under suitable conditions became infective, though of course the separated protein was not by itself infective (256 to 258). Analogous resyntheses have even been carried out using synthetic polynucleotides in place of natural RNA, though the resulting virus-like particles are not infective (259).

The x-ray study of paracrystalline TMV was first resumed in recent years by Watson (260). By applying the diffraction theory applicable to helical structures which has already been referred to (113), he demonstrated

that the TMV particle must be helical in structure; he showed further that the pitch of the helix is 23 A, and that it contained $3n+1$ subunits repeating after three turns, i.e., 69A. Watson was unable to determine n precisely, though he favoured a value in the neighbourhood of ten. More recently further studies have been made by Franklin and her collaborators (261 to 267). It has now become clear, from an analysis of x-ray photographs, technically very much improved, that the value of n is probably 16, giving 49 (or 49⅓) subunits in three turns of the helix. To establish this result the method of isomorphous replacement has been used, one mercury atom per subunit being introduced by attaching methyl mercury to a cysteine residue. The establishment of n as 16 leads to a molecular weight in the neighbourhood of 40×10^6, and it is concluded that the virus particle is made up of 2,130 subunits each having molecular weight 17,000 to 18,000.

X-ray methods have also been used to study the structure of the polymerized, RNA-free protein, derived either from monomers naturally occurring in infected plants (268, 269), or from the protein obtained by degrading TMV and removing the RNA (262). It has been found that the resemblance between these fabricated rods and natural TMV is extremely close; the former are helical arrangements of the subunits, similar or identical to that found in the natural material. This fact, coupled with the known proportions of nucleic acid (6 per cent) and protein (94 per cent) in TMV, indicated that the main features of the diffraction pattern, and the structural model derived therefrom, result from the nature and arrangement of the protein subunits.

The question then arises as to where the nucleic acid component may be situated. Some indications have been obtained from results of Caspar (270) and of Franklin (266), who used the method of isomorphous replacement. By attaching lead acetate or methyl mercury to the virus they were able to determine the phases of the equatorial reflexions, and from these to derive one-dimensional Fourier projections. These show the electron density (projected onto a radius): in one case (270) of the complete TMV particle, and in the other (266) of the repolymerized nucleic acid-free protein. The two distributions are extremely similar with the exception that the former contains a peak at a radius of 40 A while the latter contains a minimum at this radius. It is concluded by Franklin that the RNA is situated, in the intact virus, at a radius of 40 A. Both the distributions indicate a hollow of radius 20 A in the centre of the particle. We thus arrive at a general notion of the layout of the virus which may be drawn, schematically, in Figure 12. This shows the helical arrangement of subunits, each of which is 23 A thick. A thickness of 23 A would just accommodate two polypeptide chains, and evidence of infrared dichroism (271) suggests that the chains are in fact arranged radially. It will be noticed that, although the shape of the subunits has been shown purely diagrammatically, their outer ends have been so represented as to give the particle a roughened surface, a helical groove

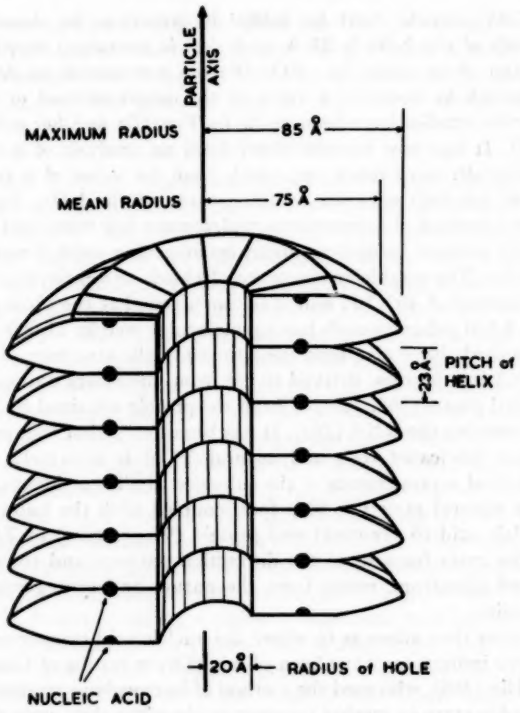


FIG. 12. Diagrammatic representation of the structure of tobacco mosaic virus. [Reproduced from Franklin, R. E., Klug, A. & Holmes, K. C., in *The Nature of Viruses*, p. 43 (J. & A. Churchill, Ltd., London, England, 292 pp., 1956).]

running between successive rows of subunits. X-ray evidence (266) suggests that a groove of this kind does in fact exist. The position of the RNA in the particle has been indicated, but as yet there is no evidence as to the way in which the individual RNA chains are arranged—if for example they form a helix, or set of helices, at the radius of 40 A, and if so what the configuration of the nucleotide chains may be.

Some recent electron microscope work (272) which may, however, be criticized on technical grounds, has been interpreted in the sense that the TMV particle is a hexagonal prism rather than a helix, but there is reason to think that the x-ray evidence, favouring the latter, is more conclusive. All in all, the helical nature of the virus particle may be regarded as certainly established. It is very remarkable that a structure so complicated should have become understood in such detail by the application of x-ray methods,

when many far smaller biological macromolecules have yielded less to a more prolonged attack by the same methods. The completeness of our understanding of the general features of the TMV structure is due, of course, to the very high symmetry of its component parts, a phenomenon itself remarkable and until recently quite unexpected in a biological, or even (according to some) a living, object.

Spherical viruses.—Results obtained by electron microscopy have by degrees made it clear that, in general, plant viruses are regular in shape and size; that many are spherical, or nearly so; and that they so closely resemble one another as to crystallize in a form no less regular in structure than the crystals of pure proteins. They may, in other words, be regarded as "molecules" in the sense of having a defined and common structure, not varying from individual to individual; and they seem to be highly symmetrical molecules at that. Crystals of plant viruses have unit cells which are cubical in shape, and though until recently the true symmetry of these crystals was unknown, it has been noticed, for example by Hodgkin (273) and by Low (274), that true cubic symmetry in a virus crystal containing one molecule per lattice point carries the implication that the individual virus particle must have cubic symmetry. This means that it must consist of at least 12 identical, and symmetrically arranged, subunits. Chemically, the plant viruses are made up of protein and of RNA, and in at least one case (turnip yellow mosaic) it has been demonstrated (275, 276, 277) that the protein forms a shell surrounding a central core of RNA. In this respect there is an analogy with the structure of TMV which, as we have indicated, is also such that the RNA occupies an interior, and the protein a mainly exterior, location.

Crick & Watson (278) have recently been led to propound a general hypothesis regarding virus structure, partly by the considerations just outlined, and partly by the results of an x-ray study by Caspar (279) of bushy stunt virus. Caspar showed that the crystals of this virus have a body centred cubic lattice, with the space group I23, the cell side being 386 A, and the unit cell containing two virus particles. This proves that the symmetry of the particle must be at least as high as that of the point-group of the lattice, viz. 23; that is to say, there must be three twofold and four threefold axes of symmetry intersecting in a point at the centre of the particle. In fact, the particle symmetry may be still higher, because single-crystal precession photographs indicated the presence of "spikes" of high diffracted intensity in reciprocal space, extending from the origin in the direction of the fivefold axes in the point-group 532, in addition to the spikes along the three- and twofold axes. The assignment of the point-group 532 to the particle would imply that it must be made up of sixty structurally identical subunits (asymmetric units), or some multiple thereof; but this cannot be proved with certainty, since 532 may only be the *pseudo*-symmetry of the particle, and its true symmetry may be lower.

Watson & Crick generalize these results by suggesting that all small vi-

ruses consist of a central core of nucleic acid, which carries the genetical information passed on by the virus to its progeny, surrounded by a protective protein shell made up (presumably for reasons of economy of manufacture) of a large number of identical, symmetrically arranged subunits. The regular aggregation of identical subunits can in general be achieved only in the form of a helix, as in TMV, or on the surface of a sphere in the manner of one of the five Platonic solids. The symmetry of these solids must correspond to one of the three cubic point groups involving rotation axes; reflexion planes are excluded, since the virus is made up of non-enantiomorphous materials. The three point groups are 23, 423, and 523, possessing 12, 24, and 60 asymmetric units respectively. Such evidence as is available suggests that small viruses really do possess the high symmetries predicted by this hypothesis, though direct confirmation of it has been published only in the case of bushy stunt virus.

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THE CHEMISTRY OF PROTEINS[1]

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In the 76 journals surveyed over the past year (October, 1955 to October, 1956) the reviewers encountered 1559 articles in amino acid, peptide, and protein chemistry. Even after the subtraction of papers dealing with proteolytic enzymes and with x-ray diffraction, the remainder is overwhelming. It is a vast and rapidly expanding field which forces selectivity and compromise on the reviewers. As the abbreviated title indicates, we have omitted papers on amino acid and peptide chemistry except as they relate directly to studies of protein structure, but we hope that the article by M. J. Coon in the 1958 volume will repair the omission. We have selected what appear to be some of the major themes in protein chemistry and discussed the relevant contributions of the year as they develop those themes.

A number of excellent books and reviews (1 to 6) which appeared during the year are referred to under the appropriate sections below.

Homogeneity

Homogeneity is a relative concept and must be operationally defined in terms of the method or methods of study used. In the case of small molecules, the organic chemist has well-defined, rigid criteria which permit positive and quantitative statements about the purity of his compounds with respect to their covalently bonded structure. In the case of proteins it is this positive and quantitative aspect that has been conspicuously lacking. As pointed out by Edsall, no single physicochemical criterion is sufficient to establish positively the purity of a protein. Innocence of contamination is impossible to prove and the accumulation of negative evidence by several independent methods only increases the probability that a given preparation is pure. With discouraging frequency, the application of a more subtle technique has revealed heterogeneity not previously demonstrable, and the work of the past year includes its share of examples. However, it should be recognized that the newer methods by which heterogeneities are exposed are in many cases discriminating to an astonishing degree. To equate them with less sensitive techniques used in the past and assume by extrapolation that resolution into new subgroups is a never-ending process may be an unduly pessi-

[1] The abbreviations for the amino acids and the conventions for presenting protein structures are those of Brand & Edsall [*Ann. Rev. Biochem.*, **16**, 223 (1947)] and Sanger [*Advances in Protein Chem.*, **7**, 1 (1952)]. Other abbreviations used in this chapter are: ACTH for adrenocorticotropic hormone; ATP for adenosinetriphosphate; DNFB for dinitrofluorobenzene; DNP for dinitrophenyl; MSH for melanocyte-stimulating hormone; TCA for trichloroacetic acid; and TMV for tobacco mosaic virus.

mistic conclusion. For example, the resolution of insulin, pure by every other available criterion, into two fractions differing by only a single amide group (296) can be looked upon as a discouraging further evidence of "microheterogeneity" or, alternatively, as evidence that there is not a statistical "population" of insulin molecules to be defined only with the aid of a statement of standard deviation. True, "insulin" is thus shown to be heterogeneous. "Insulin A" and "insulin B," however, may well be collections of identical molecules. With respect to covalently bonded structure, this seems indeed to be the case.

A prominent and exciting feature of recent work has been the accelerating rate at which data on amino acid sequence have accumulated. It may be useful to define a new term: "sequential purity," that is, homogeneity with regard to amino acid sequence and other covalently bonded structure. Complete sequences have now been reported for beef, sheep, hog, horse, and whale insulins (7); hog and sheep corticotropins (8, 9); hog glucagon (10), hog melanocyte-stimulating hormone (11, 12); beef and horse hypertensins (13, 14); oxytocin and vasopressin (297); and a number of antibiotic peptides (15 to 18). Incomplete but extensive sequence results have been obtained for ribonuclease (19 to 23), silk fibroin (24, 25), and lysozyme (26 to 29). In addition, there are many studies of N- and C-terminal residues and sequences in which stoichiometry or near stoichiometry has been obtained. An excellent discussion of results in this area will be found in the recent review articles by Anfinsen & Redfield (1a) and by Low & Edsall (2).

Studies of this kind afford positive evidence, partial or complete, of "sequential purity." None of the proteins chosen for sequence study has disappointed the investigator by failing to yield a unique sequence, uniquely reconstructed. In no case have quantitatively significant fragments been encountered that would indicate heterogeneity according to the criteria employed.

Ultimate proof of sequential purity would require the quantitative recovery of each fragment from which the sequence is inferred and agreement between the composition of the intact starting material and that of the sum of the parts. This ideal has been closely approached in the painstaking studies of Shepherd *et al.*, on hog β-corticotropin (8). Using countercurrent distribution they separated and isolated the products of tryptic digestion in over-all yield of 93 per cent. Of the six fragments resulting from chymotryptic digestion, three were recovered in theoretical yield, one in 90 per cent yield, and two in 70 per cent yield. Each of the peptides from the proteolytic digests ran as a single component on paper chromatography, showed only a single N-terminal and C-terminal group, and gave integral mole ratios on quantitative amino acid analysis. Here, then, there is no longer a question of there being a spectrum of related but distinct covalent structures. Some 70 to 90 per cent of the molecules in β-corticotropin must be identical.

Less rigorous proof of sequential purity is obtained by the successful reconstruction of a unique sequence but without a quantitative "balance sheet" of the kind discussed above. If all fragments resulting from partial degrada-

tion are examined and none are found to be incompatible with the proposed formula, the probability that the starting material is chemically homogeneous is high. Transposition of sequences cannot be ruled out, nor can the presence of alternative sequences in a small percentage of the molecules be ruled out, since minor chromatographic components may be overlooked.

The number of proteins for which detailed sequence information of this type has been obtained is small, and the proteins selected for such study represent the smallest molecules and the purest by physical criteria. Nevertheless, in this limited area, the first in which positive evidence with defined limits of error is becoming available, there is no positive support for the point of view expressed by Colvin, Smith & Cook that

> . . . it seems more correct to describe a native protein, not in terms of a finite number of chemical entities, but as a population of closely related individuals which may differ either discretely or continuously in a number of properties (30).

This concept of microheterogeneity may be, and indeed might be expected to be, valid with respect to the properties depending upon molecular configuration. A chemically homogeneous protein, in which all the molecules share the same amino acid sequence and the same covalent cross linkages, could theoretically assume a bewildering number of configurations with correspondingly different physical properties. It might be useful to characterize this type of heterogeneity as "configurational isomerism," in analogy with the terminology used for smaller molecules. Orientation toward future research, particularly with regard to the problem of protein biosynthesis, is quite different if we proceed on the assumption that we are studying chemically distinct structures rather than a spectrum, with, at best, a definable standard deviation. Until positively disproved, it would seem profitable to retain the hypothesis that proteins are unique molecules with respect to covalent structure, admitting that their complexity may permit the coexistence of many "isomeric" forms separable by appropriate methods. Whether or not such an hypothesis is valid must be determined by future studies. Here we would like to review some of the relevant studies.

Hog corticotropin.—Shepherd and co-workers have documented their careful studies on hog β-corticotropin which lead to the following formula (8, 31, 32):

Ser.	Tyr.	Ser.	Met.	Glu.	His.	Phe.	Arg.	Try.	Gly.	Lys.	Pro.	Val.	Gly.	Lys.	Lys.	Arg.	Arg.	Pro.	Val.
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Lys.	Val.	Tyr.	Pro.	Asp.	Gly.	Ala.	Glu.	Asp.	Glu(NH_2).	Leu.	Ala.	Glu.	Ala.	Phe.	Pro.	Leu.	Glu.	Phe.
21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39

As discussed above, the excellent recoveries of degradation products establish that the material is at least 70 per cent homogeneous with regard to sequence. The molecular weight, confirmed within 4 per cent by ultracentrifuge data, is 4567 (33).

The sequence reported is identical with that reported earlier except for the sequence 25 to 28, previously given as .Gly.Ala.Glu.Asp. (298). The structure previously proposed by White & Landmann (299) for hog cortico-

tropin A is identical with the above except in two minor respects. These workers give .Gly.Ala.Glu.Asp. for positions 25 to 28, as in the earlier report by Bell, and do not find the amide group in position 30. Shepherd *et al.* (8) suggest that an unusual instability in that portion of the molecule during Edman degradation may account for the difficulty in establishing the sequence 25 to 28 and that this may reflect a peculiarity of structure in that region. The absence of the amide group in corticotropin A may be attributable to differences in preparative methods. Dixon & Stack-Dunne (34) have shown that their A_1 component is probably identical with White & Landmann's A and that standing overnight at pH 11.3 converts it to their peak A_2 with the loss of one mole of ammonia. Shepherd *et al.* (31) show that β-corticotropin can be completely deamidated without loss of activity and without other apparent changes in structure by allowing it to stand in pH 9 buffer at 25° for several days. Even brief exposure to alkali during chromatography on IRC-50 columns caused considerable deamidation. These two minor discrepancies aside, the almost complete agreement of two independent laboratories considerably strengthens the case for the sequential homogeneity of hog corticotropin.

β-Corticotropin, the fraction for which the above sequence was elucidated, accounted for only about one half of the total activity of the oxycellulose-purified starting material. The remainder was distributed among seven other components clearly separable by countercurrent techniques. Surprisingly, all of these peaks had essentially identical biological activity so that they are functionally indistinguishable from β-corticotropin. There were no qualitative differences in amino acid composition and only slight quantitative differences (not specified). The only clear difference observed was that on peptic digestion, which only yielded three active products from β-corticotropin, the other peaks yielded six to eight products. The nature of the differences between all of these multiple components is not yet determined. One minor fraction (α_4), however, appears to be simply β-corticotropin in which the C-terminal carboxyl group became esterified during isolation and separation. The multiplicity of components found by Shepherd *et al.* (31) is to be contrasted with the results of White (300), who observed only one active component on ion-exchange chromatography of similarly prepared starting material. Whether some of the other minor components arise during the preparative procedures or whether they represent different forms present in the pituitary gland remains to be determined.

Sheep corticotropin.—Li and co-workers elucidated the following structural formula for sheep α-corticotropin (9), which is in perfect agreement with quantitative analyses on the intact starting material reported previously (301):

Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Try	Gly	Lys	Pro	Val	Gly	Lys	Lys	Arg	Arg	Pro	Val
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Lys	Val	Tyr	Pro	Ala	Gly	Glu	Asp	Asp	Glu	Ala	Ser	Glu	Ala	Phe	Pro	Leu	Glu	Phe
21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39

There are, in addition, two amide groups not yet localized. Without quantitative values for recovery of peptide fragments, a quantitative statement regarding sequential homogeneity cannot be made. However, the failure to find any peptide components with sequences inconsistent with the proposed structure must mean that little if any sequential isomerism occurs.

It will be noted that the above structure differs from that of hog corticotropin in only three minor respects: (*a*) the sequence in positions 31 to 32 is .Ala.Ser. instead of .Leu.Ala.; (*b*) the sequence in positions 25 to 28 is .Ala.Gly.Glu.Asp. instead of .Asp.Gly.Ala.Glu., as in β-corticotropin, or .Gly.Ala.Glu.Asp., as reported for corticotropin A; and (*c*) the presence of two amide groups instead of one, as in β-corticotropin, or none, as in corticotropin A. The evidence regarding structure-function relationships is discussed later, but it can be pointed out parenthetically that the differences in sequence are confined to the residues beyond position 24, which do not appear to be essential for corticotropin activity.

Preliminary studies of beef corticotropin by White & Peters (35) and by Li & Dixon (36) show that the N-terminal sequence and the C-terminal residue are the same as in sheep and hog corticotropins. Quantitative amino acid analyses by Li & Dixon lead to an empirical formula identical with that for sheep corticotropin, and differing from that of hog corticotropin in having one more serine residue and one less leucine residue. The apparent identity of the hormones in sheep and cattle, if corroborated by studies of internal sequences, would be consonant with the closer phylogenetic relationship of these species.

Hog melanocyte-stimulating hormone (*MSH*).—The following structural formula, proposed by Harris & Roos (11), was obtained for a sample purified from hog posterior pituitary glands by the method of Porath *et al.* (302):

Asp.	Glu.	Gly.	Pro.	Tyr.	Lys.	Met.	Glu.	His.	Phe.	Arg.	Try.	Gly.	Ser.	Pro.	Pro.	Lys.	Asp.
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

The sequence of the first ten residues was determined by successive application of the Edman degradation, indicating the potential of this method when, as in this case, the same amino acid residue is not repeated within a short distance along the chain. Again here, none of the peptide fragments resulting from proteolytic degradation yielded a sequence inconsistent with the proposed structure, and the over-all amino acid analysis is completely compatible with the structural formula. The results of Geschwind, Li & Barnafi, using similar methods, confirm this structure in every respect (12) The minimum molecular weight is 2177. The concurrence in two laboratories means that the above structure is almost certainly correct as it stands, and it becomes necessary to rationalize the conflicting amino acid analyses, previously reported from three laboratories, none of which are in agreement with each other or with the above structure (302 to 304). It is possible that there are several melanocyte-stimulating hormones in the posterior pituitary. On the other hand, the most active preparation, prepared by Benfey & Purvis (304)

by a 180-transfer countercurrent distribution, gave an amino acid analysis most nearly approximating that indicated by the present formula. The preliminary qualitative analyses by Porath *et al.* (302) failed to reveal methionine and showed some alanine and valine. More recent quantitative studies by Roos, presumably on material purified in a similar manner, completely agreed with the above sequence (37). Winnick *et al.* find more polypeptide material in extracts of the posterior pituitary than can be attributed to the known hormones. Contamination by some of this material may account in part for the disagreement in earlier studies (38).

The intriguing point about this structure is that the heptapeptide sequence in positions 7 to 13 coincides exactly with the heptapeptide sequence in positions 4 to 10 of the corticotropins. It is now well established that the low melanocyte-stimulating activity of corticotropin is not attributable to contamination but is intrinsic to the corticotropin molecule (31, 39). As pointed out by Harris & Roos (11), this example of "shared" activities between molecules with partially "shared" amino acid sequences is similar to that of the structurally and functionally related oxytocic and vasopressor peptides of the posterior pituitary.

Glucagon.—Bromer *et al.* reported the following complete amino acid sequence for hog glucagon (hyperglycemic-glycogenolytic factor), first crystallized in their laboratory last year (10):

$$\underset{1}{\text{His}}.\underset{2}{\text{Ser}}.\underset{3}{\text{Glu(NH}_2\text{)}}.\underset{4}{\text{Gly}}.\underset{5}{\text{Thr}}.\underset{6}{\text{Phe}}.\underset{7}{\text{Thr}}.\underset{8}{\text{Ser}}.\underset{9}{\text{Asp}}.\underset{10}{\text{Tyr}}.\underset{11}{\text{Ser}}.\underset{12}{\text{Lys}}.\underset{13}{\text{Tyr}}.\underset{14}{\text{Leu}}.\underset{15}{\text{Asp}}.\underset{16}{\text{Ser}}.\underset{17}{\text{Arg}}.\underset{18}{\text{Arg}}.\underset{19}{\text{Ala}}.$$

$$\underset{20}{\text{Glu(NH}_2\text{)}}.\underset{21}{\text{Asp}}.\underset{22}{\text{Phe}}.\underset{23}{\text{Val}}.\underset{24}{\text{Glu(NH}_2\text{)}}.\underset{25}{\text{Try}}.\underset{26}{\text{Leu}}.\underset{27}{\text{Met}}.\underset{28}{\text{Asp(NH}_2\text{)}}.\underset{29}{\text{Thr}}.$$

The constituent peptides obtained by proteolytic breakdown were recovered in yields exceeding 50 per cent, no incompatible sequences were encountered, and the empirical formula from quantitative amino acid analysis checked with the structural formula. In this case, then, the preparation is at least 50 per cent sequentially pure. The minimum molecular weight is 3482.

The structure bears little relation to that of insulin, although the .Phe.-Val.Glu(NH_2). sequence echoes the Phe.Val.Asp(NH_2). sequence at the N-terminus. The predominance of amino acids with functional groups in the side chains is noteworthy.

Insulin.—Sanger and co-workers have now determined the structure for insulin from five species (7, 305). The differences were limited to positions 8 to 10 of the A chain. The rest of the A chain and the entire B chain appear to be identical in all the species examined. The sequences observed in positions 7 to 10 were: .$CySO_3H$.Ala.Ser.Val. (beef); .$CySO_3H$.Thr.Ser.Ileu. (pig); .$CySO_3H$.Ala.Gly.Val. (sheep); .$CySO_3H$.Thr.Gly.Ileu. (horse); and .$CySO_3$-H.Thr.Ser.Ileu. (whale). This tetrapeptide sequence in the A chain is flanked by two half-cystines joined through an intrachain disulfide bridge. Because of the occurrence of this 20-membered disulfide ring in insulin and similar 20-membered rings in oxytocin and vasopressin, the possibility that this

structural feature might be intimately connected with biological activity had to be considered. It now appears that the nature of the amino acids within the ring may not be specifically related to function, although the ring structure itself may be important. The loss of insulin activity on reduction is compatible with the latter possibility. Ressler has recently synthesized the cyclic pentapeptide amide corresponding to oxytocin less its "tail" [.Pro.Leu.Gly-(NH_2).], that is, the cyclic disulfide ring itself. While the product retained some oxytocic and milk-ejecting properties, the activities were very low and all of the avian-depressor activity was lost (40). Evidently the 20-membered ring is not sufficient to account for the biological activity, although it may prove to be necessary.

A question regarding the sequential homogeneity of insulin has been raised by Grassmann *et al.* (41) which requires discussion. Their quantitative analyses, like the earlier ones of Harfenist (296), show one less valine and one less isoleucine than indicated by Sanger's formula. By use of continuous paper electrophoresis they separated the A and B chains, after performic acid oxidation, with over-all recoveries of 97.6 per cent. In the A chain they found only one isoleucine residue instead of two and only three valine residues instead of four for an insulin molecule of 12,000 mol. wt. unit. These results are incompatible with a molecular weight of ~6000 and lead Grassmann *et al.* to suggest that not all A chains are identical. They propose that Sanger's positions A2 and A3 may actually be a single position alternatively occupied by valine or isoleucine; sequence studies were not reported.

Harfenist attributed her low yields to the expected resistance of the Ileu.Val bond to acid hydrolysis (296). By extending the time of hydrolysis to 96 hr. the yields were increased to values agreeing within a few per cent with the integer values in Sanger's formula. Grassmann *et al.* extended the time of hydrolysis to 48 hr. and although this did not significantly increase their valine recovery, isoleucine recovery increased to 1.40 moles/12,000. This value is rather high if the true figure is only 1.0. Perhaps the most telling point is that Sanger's analysis of peptide fragments derived from the A chain included at least eight different components containing the .Ileu.Val. sequence. This sequence could not arise from the structure proposed by Grassmann, *et al.*

Because the recoveries of the fractionated A and B chains in Sanger's original sequence studies were low and because quantitative data on recoveries of individual peptide fragments were not presented, judgment had to be reserved on the question of whether all insulin molecules shared the amino acid sequence of the material actually studied. In the more recent species studies the fractions were separated by ionophoresis, thus eliminating the losses associated with precipitation. Furthermore, aside from the minor discrepancies discussed above, the amino acid analyses by Grassmann *et al.* on the separated chains obtained in almost quantitative yield, satisfy the criterion of agreement between empirical formula and structural formula for the two chains individually. Sequential isomerism is not absolutely ruled out, but the failure

to find any incompatible sequences in the monumental number of peptides examined in the study of five different insulins, and the unambiguous localization of disulfide bridges in beef insulin (306), speak strongly for sequential homogeneity.

Hypertensins.—A pressor peptide produced by the action of rabbit renin on ox serum was purified by Elliott & Peart (13, 42) and shown to have the following structure:

Asp.Arg.Val.Tyr.Val.His.Pro.Phe.His.Leu.
1 2 3 4 5 6 7 8 9 10

Skeggs and co-workers (14, 43 to 46) have characterized two closely related peptides in horse serum, both of which have pressor activity when tested in whole animals. Hypertensin I, which is not active when tested in isolated tissue systems, is converted by a partially purified "hypertensin-converting enzyme" isolated from horse plasma to hypertensin II, which is active both *in vivo* and *in vitro*. Hypertensin II is presumed to be the directly active principle and its structural formula has been shown to be as follows·

Asp.Arg.Val.Tyr.Ileu.His.Pro.Phe.
1 2 3 4 5 6 7 8

The conversion of form I to form II is associated with the release of a dipeptide, His.Leu. Assuming this is released from the C-terminus of hypertensin I, it will be seen that the sequence for the beef hypertensin of Elliott & Peart and that for horse hypertensin I are identical except for the substitution of isoleucine for valine at position 5. The marked inhomogeneity of some hypertensin preparations may indicate the presence of additional active peptides or unrelated contaminants (47). The chemical characterization of these pressor principles represents a landmark in the development of an area that has tended in the past to lack definition.

Ribonuclease.—Parallel studies by Redfield & Anfinsen (19, 20) and by Hirs, Bailey *et al.* (21, 22, 23) have led to the simultaneous publication of compatible partial structural formulae for bovine pancreatic ribonuclease. While there remain a number of gaps in which the sequence is not yet known, both groups have established the order of alignment of the large peptide fragments obtained by enzymatic degradation. Redfield & Anfinsen employed a device which may be valuable as a general procedure for sequence studies on larger proteins. The performic acid-oxidized protein was treated with DNFB or, in later studies by Anfinsen, Sela & Tritch, with carbobenzoxychloride (48). With the lysine ε-amino groups thus masked, trypsin attacked only at the four arginine residues, yielding the expected five peptide fragments. The isolation of arginine-containing "overlap" sequences produced by other degradative procedures (pepsin digestion, partial acid hydrolysis) then permitted the proper alignment of these five peptides. The carbobenzoxylation technique was preferred since the peptide fragments, after removal of the carbobenzoxy groups with formic acid-HBr, were more easily separated than the DNP-peptides.

Hirs, Bailey *et al.* studied the peptides derived from peptic, tryptic, and chymotryptic digestion of oxidized ribonuclease. The complex mixtures of peptides were fractionated with remarkable resolution and in high yield by ion exchange chromatography on 150 cm. columns of Dowex 50-X2. For example, 13 fragments derived from tryptic hydrolysis were partially or completely separated in yields of 50 to 100 per cent in a single chromatogram. The demonstrated power of the ion exchange methods used in this work highly recommends them for future studies on complex peptide mixtures. The quantitatively defined recoveries permit positive statements regarding homogeneity, at least of the segments of the molecule so far examined.

Silk fibroin.—Lucas, Shaw & Smith (24) published results of quantitative amino acid analyses and sequence studies on the previously reported precipitate formed during chymotryptic digestion of *Bombyx* silk fibroin. This precipitate, representing 62 per cent of the total silk fibroin nitrogen, mol. wt. about 4000, had the empirical formula Gly_{29}, Ala_{20}, Ser_9, Tyr_1. No Gly.Gly could be found in partial hydrolysates, indicating that glycine must occupy alternate positions. Taking advantage of the N to O acyl shift at serine residues, it was possible to isolate 36 per cent of the material as: Ser.Gly.Ala.-Gly.Ala.Gly. These results, together with results on the sequences of peptides in partial hydrolysates and of DNP-peptides from the N-terminus (DNP.-Gly.Ala.Gly.Ala.) led to the following formulation: Gly.Ala.Gly.Ala.Gly.$[Ser.Gly.(Ala.Gly)_n]_8$Ser.Gly.Ala.Ala.Gly.Tyr, where n is, on the average, and, usually, two. Waldschmidt-Leitz & Zeiss (307), studying a fraction apparently prepared in a similar fashion, reported a different empirical formula: Gly_{21}, Ala_{14}, Ser_7, Tyr. The sequences in several di- and tripeptides (some of which are incompatible with the structure of Lucas, *et al.*) were compatible with the following formula: $(Gly.Ala.Ser.Gly.Ala.Gly)_7$.Tyr.

Bombyx silk fibroin (BSF) and *Tussah* silk fibroin (TSF) are alike in that over 90 per cent of the amino acids consist of glycine, alanine, serine, and tyrosine (308). In the case of BSF, sequence studies have shown a regular alternation of glycine with other residues along the chain, in agreement with the predictions of Marsh, Corey & Pauling (309) from x-ray studies. The relative preponderance of glycine over alanine in BSF is reversed in TSF, and the x-ray evidence would be compatible with alternation of alanine residues along the chain. However, studies by Kay, *et al.* on the peptides in partial acid hydrolysates of TSF show that the alanine residues are bunched to a striking degree (25); almost 35 per cent of the alanine in the protein was accounted for in the form of Ala.Ala, Ala.Ala.Ala, Ala.Ala.Ala.Ala, and Ser.Ala.Ala.-Ala; another 25 per cent of the alanine remained in an insoluble residue representing only 10 per cent of the original TSF. Since approximately 50 per cent of the amino acid residues in this "core" were alanine residues, the bunching of alanine in the "core" is even more striking than it is in the soluble portion studied. Thus the difference between the silk made by wild species of silkworm (TSF) and that made by the cultivated variety (BSF) is quite dramatic. The former is characterized by a preponderance of polyalanine se-

quences; the latter by regular alternation of glycine, a large part of it in the repeating hexapeptide, Ser.Gly.Ala.Gly.Ala.Gly.

To these thoroughly investigated cases can be added a large number of examples in which determination of terminal groups, terminal sequences, or specific internal sequences suggests some degree of homogeneity, at least with respect to the portions of the molecules studied [TMV (49, 50, 57, 63); plasma proteins (51 to 56); hemoglobin (57, 58, 59); casein (60); pepsin (61); papain (62); flagellin (64); bacillomycin (65); grain proteins (66); β-lactoglobulin, salmine, and others (63)].

The fact that a chemically pure protein can be isolated from a tissue does not necessarily rule out the possibility that that tissue produces a broad spectrum of related protein molecules. There may be a high degree of selectivity in the isolation and purification procedures with "survival of the purest." Occasionally it is feasible to check this possibility by direct examination of crude extracts obtained with a minimum of handling. For example, Martin & Porter (310) and Hirs, Moore & Stein (311) have shown that chromatography of the first crude extracts of raw beef pancreas show the same two ribonuclease components found in the purest crystalline material, and only those two components. Even here the acid extraction may already have discriminated against related structures.

Conversely, the isolation of multiple related proteins does not necessarily prove that these are all bona fide products of protein biosynthesis. Zymogens and their closely related active derivatives, fibrinogen and fibrin, hypertensinogen and hypertensin, and prothrombin and thrombin, are examples in which it seems likely, even though not rigidly established, that the synthetic machinery makes only the precursor molecule and that the products are formed from them subsequently. Furthermore, there is the real possibility that procedures used in isolation and purification may contribute to inhomogeneity.

The homogeneity of the starting material must be considered. For example, Brown, Sanger & Kitai (305) suggest that the finding in beef insulin samples of small and variable amounts of peptide sequences characteristic of sheep insulin and of pig insulin may be attributable to contamination at the slaughter house. Even the use of pooled samples from many individuals of the same species can introduce inhomogeneity, as discussed below.

Modification attributable to handling and storage is known to contribute to inhomogeneity in a number of instances. For example, chromatography of growth hormone by the relatively mild procedure of pH gradient elution from diethylaminocellulose columns has been shown to give rise to a new component (67). Hirs [quoted in (68)] has shown that chymotrypsinogen, homogeneous on IRC-50 columns, develops asymmetry on storage. Loss of amide groups has been encountered during purification or storage of several proteins. The rapid deamidation of corticotropin by weakly alkaline buffers such as are used in column chromatography has been discussed above. The basis for the derivation of two more acidic components from lysozyme during

storage, while not conclusively established, may again be deamidation (312). Raacke has shown that one month's storage of lyophilized ribonuclease causes some conversion of the A form to the B form (68). Moreover, it appears that different preparations of crystalline ribonuclease, while they all contain both A and B, can show widely different proportions of the two forms. Tanford & Hauenstein, studying a preparation with approximately equal amounts of A and B, have shown that according to titration data (69), they differ by only one carboxyl group. Attempts to demonstrate other differences failed, including viscosity and light-scattering studies. Of particular interest are the observations made with the sensitive differential spectrophotometric technique. They indicate that the phenolic hydroxyl groups in ribonuclease A and B must be identically situated and identically bonded. These results provide an adequate explanation for the presence of two components in ribonuclease and do not require the postulation of differences in the state of oxidation of sulfhydryl groups (313), although active reduction may indeed modify the native molecule and increase the heterogeneity (70). Apparently, then, storage can lead to deamidation of the B component, and different preparative procedures may cause greater or lesser degrees of deamidation. On the other hand, the studies of Hirs, Moore & Stein (311) on crude extracts, and on extracts exposed to acid-alcohol for varying periods of time, strongly suggest that some of the B form is resident in the cell. The case of insulin A and B is quite analogous. Both are present in all beef insulin preparations but, again, in varying proportions. Since the proteins discussed here are almost invariably prepared from large pools of starting material and since genetically related proteins may differ only in very minor respects, such as number of amide groups (see p. 387), it would be of interest to examine samples derived from single pancreases, single eggs, or, if possible, single pituitary glands.

In one instance, at least, it appears that our criteria for homogeneity may be too severe. Ogston & Tombs have re-examined the theoretical basis of the variable-solvent solubility test and show, first, that a pure protein that can form two different solid phases will lead to a discontinuity and, second, that this can be differentiated from two independent proteins by obtaining data at several different levels of protein (71). Zittle has shown that α-lactalbumin, homogeneous in the ultracentrifuge and by electrophoresis, can exist in two forms with markedly different solubilities in ammonium sulfate. The "insoluble" form, however, is readily converted to the "soluble" form simply by dialysis. The author attributes the apparent inhomogeneity indicated by solubility tests to changes in the molecule accompanying the binding of anions (72).

Electrophoretic evidence of inhomogeneity must be interpreted with caution, particularly in solutions of low ionic strength where anomalies are likely to occur. Two important causes of "artifact" splitting of a single component are: (*a*) interaction with ions, and (*b*) association-dissociation reactions. Some proteins are able to bind a few specific ions very tightly, so that

the concentration of the free ion will be practically zero and the interchange of bound ions between protein molecules will be extremely slow. In this case, if not all of the available sites are occupied by the ion, a heterogeneity will be apparent on electrophoresis. This is probably the explanation of the two boundaries seen with zinc-free insulin, which give place to a single boundary on binding one zinc ion per mole of protein (73). Such a mechanism may also be responsible for the disappearance of the electrophoretic heterogeneity of β-lactoglobulin when two moles of dodecyl sulfate are bound (314). Similarly, binding of fatty acids or other anions may be the cause of the splitting of the serum albumin peak in some cases[3] (74). Interaction of proteins with ions is usually a very fast process, but may result in structural changes which are only slowly established or reversed (isomerization); this is another way in which binding of ions can bring about heterogeneity. The isomerization of bovine serum albumin at low pH (78, 79), or higher pH in the presence of acetate (80), is discussed below. Binding of dodecyl sulfate to bovine serum albumin (315) and *n*-octylbenzene-*p*-sulfonate to β-lactoglobulin (81) has a similar effect.

Finally, the possibility of association-dissociation reactions should be noted. Dissociation reactions may be responsible for the splitting of human hemoglobin into several components observed at low ionic strength and elevated temperatures (82). Special caution is thus advisable in interpretation of heterogeneity at low pH, low ionic strength, or in the presence of certain special ions. Absence of enantiography in the two limbs of the Tiselius apparatus is strongly indicative of interaction with buffer ions.

On the other hand, real electrophoretic heterogeneity is suggested when the components can be isolated and shown to give a single boundary in a second electrophoresis run under conditions identical with those used in the first. Binding of Ca^{++} is apparently the cause of the heterogeneity of trypsin described by Timasheff, Sturtevant & Bier (83). In this case, however, the isolated fast component was homogeneous in a second electrophoresis,

[3] The presence of serum components with electrophoretic mobility greater than that of albumin has been noted by many laboratories over the past 10 years. In some cases (lipemic plasmas, postheparin plasmas) this fast component may have been due to the binding of fatty acids to lipoproteins, as clearly shown by Gordon (339) and confirmed in several laboratories (75, 76) but in others, such an explanation is not likely. Williams & Grabar (340), using the immunoelectrophoresis technique, presented clear evidence for a prealbumin which they designated the "rho"-component. Schultze, Schonenberger & Schwick (77) have now purified and characterized a component, which comprises only about 0.5 per cent of the total serum proteins, which has a mobility 50 per cent greater than serum albumin, 10 times the tryptophan content, 1.12 per cent hexose, and definitely different immunochemical properties. The molecular weight by light-scattering is 61,000 and the $S_{20,w} = 4.2$. The absence of phosphate and the failure to stain with Sudan black appear to rule out the possibility that this prealbumin is simply a lipoprotein accelerated by binding of anions. Baudouin *et al.*, while they did observe a lipide-rich prealbumin, also noted the presence of a second prealbumin component which did not stain heavily for fat (76).

showing that there are actually two species of the protein. They have identical enzymatic activities but differ in their Ca^{++}-binding ability. γ-Globulin also splits into several components at low ionic strength and here again the isolated fastest component gave only a single peak when rerun (84). Smithies (85) has shown that the resolving power of zone electrophoresis in starch gels is in many cases superior to that of the Tiselius apparatus. By combining paper electrophoresis with a second dimension in starch he was able to resolve human serum proteins into over 15 components (86). Raacke, using starch block electrophoresis, has demonstrated heterogeneity in chymotrypsinogen, lysozyme, and bovine serum albumin (68).

The recent comprehensive reviews by Moore & Stein on column chromatography and by von Tavel & Signer on countercurrent distribution indicate the increasing range of applicability of these techniques and their remarkable resolving power for peptides and proteins (1b, 1c). Many papers report inhomogeneities demonstrable with these sensitive methods (87 to 92). Of particular interest is the detailed description by Peterson, Sober and co-workers of their use of cellulose derivatives as ion-exchangers in protein fractionation (93, 94). The columns have remarkably high capacity (700 mg. protein nitrogen on a single 47×2.5 cm. column) and excellent recoveries are obtained.

A survey of recent results shows, then, that many "pure" proteins can be further resolved, particularly by the application of newer, highly discriminating techniques, such as column chromatography. Before the significance of such resolution can be evaluated, particularly its implications with respect to protein biosynthesis, the individual components must be carefully examined to establish the basis of the resolution, and possible contribution of factors such as those discussed above must be considered.

Mutability of Protein Structure

Closely related to the question of homogeneity is the question of whether the protein structure as elaborated by the cell is fixed and immutable or whether alternative structures, more or less closely related, can be synthesized.

Gross & Tarver (95) have added to the case for mutability by showing rather conclusively that ethionine-C^{14}-ethyl is incorporated into the proteins of *Tetrahymena pyriformis* and, by demonstration of ethionine-containing peptide fragments in partial acid hydrolysates, that the incorporation represents true peptide bond formation.

The incorporation of 7-azatryptophan into the protein fraction of a tryptophan-requiring *E. coli* mutant was reported by Pardee, Shore & Prestidge (96). Peptide-bond formation was not explicitly demonstrated but the extent of incorporation (0.5 per cent) corresponded to the tryptophan incorporation expected during equivalent growth. A number of inducible and constitutive enzymes failed to increase in the presence of the analogue but serine deaminase and ureidosuccinic acid synthetase activities were shown to increase.

Black & Kleiber found incorporation of norleucine into casein in lactating

cows injected intravenously with the C^{14}-labeled amino acid. They calculated that at least 4.7 mg. of radioactive norleucine (chromatographically isolated) were present per 100 gm. of casein, whereas analyses for norleucine in normal casein were negative although sufficiently sensitive to detect as little as 1.6 mg. per 100 gm. (97). Control experiments appear to rule out adsorption of radioactive norleucine during preparation of the casein. Studies on partial hydrolysates are being undertaken to establish that the incorporated norleucine is part of the casein peptide chain.

Munier & Cohen showed that *E. coli* will continue to grow, linearly but not exponentially, after the addition of *p*-fluorophenylalanine to the medium and that highly significant amounts of the analogue are incorporated into protein (98). Incorporation of β-2-thienylalanine was also demonstrated. Moreover, it was possible to show continuing production of the induced enzyme β-galactosidase in the presence of *p*-fluorophenylalanine at a rate comparable to that in controls when referred to the rate of increase in cell mass. On the other hand, the proteins produced during growth in the presence of β-2-thienylalanine, 4-methyltryptophan, or 5-methyltryptophan had no β-galactosidase activity. The authors suggest that the toxicity of the analogue is based on the synthesis of structurally and functionally abnormal proteins containing it.

These studies strongly suggest that "abnormal" amino acids, closely related in structure to the naturally occurring molecules, can substitute for the latter in the fabric of proteins. Before this conclusion can be rigorously drawn it must be demonstrated: (*a*) that the analogue can be incorporated into a well-characterized, pure protein; (*b*) that such incorporation is through true peptide bond formation. The presence of the analogue in the gross mixture of TCA-precipitable proteins might, for example, only reflect incorporation into incomplete, "aborted" protein molecules, further synthesis being blocked by the abnormal structure. The first criterion is satisfied by the casein studies. The studies showing increases in enzyme activities in the presence of analogues must be supplemented by direct isolation of the enzyme proteins containing analogue. The second criterion is satisfied by the ethionine studies. Both criteria have not yet been fulfilled for any one protein, but the evidence taken together makes it seem very likely that the biosynthetic machinery has compromised and made a slightly "abnormal" protein. The fact that growth of the organisms is eventually arrested indicates that the compromise is not a very happy one.

The postulate that the cell faithfully and rigorously produces proteins with unvarying amino acid sequences does not necessarily imply that perversions cannot be induced under unnatural conditions. On the other hand, the fact that the synthetic mechanism will accept a structurally related but different molecule is circumstantial evidence suggesting that other variations, such as isoleucine-valine substitutions, may occur. This has yet to be demonstrated explicitly.

Genetic Determination of Protein Structure

Of great importance is the demonstration in three new cases that individual mammals can produce proteins of a given class in slightly different forms and that the differences are genetically determined and inherited. Evans, King and co-workers have shown that there are two types of sheep hemoglobin, clearly distinguishable by paper electrophoresis (99). Individual animals may have either type A hemoglobin alone, type B alone, or a mixture of A and B. Breeding of two "type A" animals yielded only "type A" progeny: breeding "type A" and type "AB" animals yielded "type A" and "type AB" progeny in about equal numbers. The results of such breeding studies and of a population survey of over 1900 sheep are compatible with genetic determination by two alleles, each controlling the formation of one kind of hemoglobin.

Aschaffenburg & Drewry have confirmed the presence of two electrophoretically distinct β-lactoglobulins in cows' milk but find that some cows produce only the faster migrating β_1-lactoglobulin, others only the somewhat slower β_2-lactoglobulin, and still others a mixture of the two (100). The β_1- and the β_2-forms were crystallized from milk samples of individual animals and were found to have distinctly different crystalline forms and measurably different isoelectric points and solubilities; studies of inheritance are under way but not yet reported. However, the fact that a number of sets of one-egg twin heifers had identical β-lactoglobulin patterns strongly suggests that there is the same sort of genetic basis here as in the case of the sheep hemoglobins discussed above.

Smithies & Walker find that human sera fall into three distinct categories based on the nature of their haptoglobin content (101, 102). Haptoglobin, readily identified by its remarkable affinity for hemoglobin, runs as a single component in the α_2-globulin region on paper electrophoresis, but splits into a number of components on starch gel electrophoresis by the method described by Smithies (85). One group of sera (haptoglobin 1-1 type) contains a "fast" α_2-haptoglobin missing from the other two (haptoglobin 2-1 and 2-2 types), each of which shows a group of three hemoglobin-binding bands behind β-globulin but with definitely different group mobilities. Although the data are not yet extensive, studies on inheritance are again compatible with genetic determination in a classical Mendelian pattern. Matings of two "1-1 type" or two "2-2 type" individuals yield offspring all with sera of the same type as the parents. On the other hand matings of two "2-1 type" individuals yield all three types of offspring, indicating that this is the heterozygous type. The apparent absence of the "fast" α_2-haptoglobin from these individuals is therefore noteworthy.

To this list of more recent examples may be added the well-studied case of the electrophoretically distinguishable forms of human hemoglobin. Normal adult hemoglobin (A) and sickle-cell hemoglobin (S) have been clearly shown to be determined by single alleles at the same locus in a manner

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analogous to that for the proteins discussed above. The amino acid compositions are identical within the limits of error of the methods used and both are distinctly different from fetal hemoglobin (103). Because of the size of the molecule, differences limited to a few residues would not be detected. There is general agreement that they are identical with respect to N-terminal residues although there is disagreement on whether the correct absolute value is four or five valine residues (104, 105, 106). While the quantitative results are not readily interpretable because of the multiple-chain structure of hemoglobin, treatment with carboxypeptidase at least revealed no qualitative differences (107). Re-examination by the more sensitive and more specific amperometric procedure recently reported by Benesch, Lardy & Benesch revealed eight sulfhydryl groups per mole in hemoglobin A (108), in agreement with the previous work of Ingram (316). This value is confirmed by Hommes, Drinkwaard & Huisman, who find the same value for hemoglobin S (109). It should be noted that these results correct the low half-cystine content previously reported (317).

The basis for the separation of hemoglobins A and S is a difference in electrophoretic mobility corresponding to a charge difference of three (318). Recent studies by Ingram appear to localize the difference to one segment of the molecule (110). Tryptic digests of the two forms of hemoglobin were "fingerprinted" by separation on large sheets of filter paper using electrophoresis in one direction and chromatography in the other. Since there are approximately 60 lysine plus arginine residues in hemoglobin, some 60 fragments would be expected if all the chains were of different structure. Actually, as was anticipated, only about 30 peptide components were found in the digests, implying sequential identity for the two half-molecules. The "fingerprint" patterns for A and S were indistinguishable except for a single component which was uncharged in the digests of hemoglobin A but positively charged in digests of hemoglobin S. The higher positive charge of this key peptide could result from the presence of additional basic amino acids or a deficiency in negative charges. The previous studies of Scheinberg, Harris & Spitzer (319) favor the latter possibility. There may, of course, be differences in other peptide fragments as well but not of such a nature as to permit separation by the methods used.

The four examples cited show that in addition to the well-recognized species differences between proteins, there are distinct differences between the proteins produced by individual members within a species. In a sense, then, there are families of closely related proteins. However, the criteria distinguishing these closely related proteins, at least in three cases, divide them sharply into just two classes. A given individual synthesizes one of the two types or both types, not a broad spectrum of molecules. This is very different from a continuum of proteins such as might be produced by a "permissive" biosynthetic mechanism. Studies of this kind suggest a remarkable specificity of protein biosynthesis on a "one-allele-one protein" basis. Unresolved β-lactoglobulin, that is, material prepared from pooled samples of cows' milk,

has been previously shown by a number of workers to be inhomogeneous (cf. 320), consisting of two major electrophoretic components. This example of "microheterogeneity" is now beautifully accounted for by the work of Aschaffenburg & Drewry. Indeed, whereas the inhomogeneity of unresolved material could be readily demonstrated by solubility studies, the separately crystallized β_1- and β_2-lactoglobulins are reported to be pure according to the rigid variable-solvent solubility test [Ogston & Tombs (71)].

Many examples of direct gene-enzyme relations have been demonstrated in microorganisms but in very few cases has any information been obtained as to the nature of the changes in protein structure which correlate with the changes in genetic pattern (111). The fundamental importance of the examples cited above lies in the ready availability to the protein chemist of the variant proteins in apparently pure form. If the mechanisms for converting genetic information into a specific protein structure are fairly direct, systematic study of the structural differences, differences which must reflect differences in allelic genes, may eventually yield valuable information bearing on the molecular basis of inheritance and on protein biosynthesis.

In this connection is is of interest to speculate on the significance of the reported complete absence of "fast" α_2-haptoglobin from the sera of heterozygous individuals. The pattern here differs from that in the other cases, in which the heterozygous animal produces the proteins corresponding to both alleles. The observation itself probably deserves further study in view of the difficulties introduced by overlapping of haptoglobin and other globulins in this region. Humans who are heterozygous with respect to the genes determining hemoglobins A and S do not have equal amounts of these proteins in their red cells. Hemoglobin A tends to be favored in every instance. In heterozygous cows the production of β_1-lactoglobulin appears to be favored, according to the published electrophoresis diagrams (100). The difference in production rates for the haptoglobin in the heterozygote may be simply an extreme example of this kind of preferential formation or survival. Whether there is a very small amount of "fast" α_2-haptoglobin in the haptoglobin 2-1 type of individual, or none at all, the analogy with classical dominance and recessiveness at a grosser phenotypic level is clear. Dominance and recessiveness interpreted at the molecular level may be reflections of allelic gene configurations conducive to more rapid or less rapid protein synthesis. On the other hand, the pathway from gene structure to phenotypic manifestation may still be lengthy and tortuous, even at this level of the production of individual protein molecules. Furthermore, the differences in proportion observed may result from differences in stability and biological half life, not from differences in rate of synthesis.

A final and important conclusion growing out of consideration of studies like those discussed above is that pooled tissues or body fluids from large numbers of animals do not constitute an ideal starting material for the preparation of pure proteins. A demonstration of heterogeneity in proteins prepared from such starting materials may have a significance quite different

from a demonstration of heterogeneity in material prepared from tissues of an individual animal. In the many instances in which pooled starting material is almost always used—e.g. milk proteins, serum proteins, pituitary proteins, egg proteins, and pancreatic proteins—a re-evaluation of demonstrated heterogeneities may be in order.

Cyclic and Branched Structure

Tobacco Mosaic Virus (TMV).—Positive evidence for a new kind of intramolecular covalent linkage in proteins has been reported by Braunitzer (112). It has been known for some time that the N-terminal residue in TMV protein is masked and unavailable for reaction with DNFB in the native state. Very mild treatment with 5 per cent TCA suffices, however, to completely expose the free amino group of a proline residue (1 mole/17,000 gm.) (321). Now Braunitzer has shown that reaction of the virus protein with hydroxylamine will also expose the N-terminal proline under conditions which do not expose any other new end-groups, either in TMV or in a number of other proteins. These observations suggest that the proline amino group is blocked through formation of a peptide bond with the ω-carboxyl group of a dicarboxylic amino acid. Proof of this was obtained by exhaustive digestion with a complex protease-peptidase mixture from *Aspergillus oryzae*, treating the digest with DNFB, and isolating a DNP-peptide containing proline and aspartic acid. Mild treatment with TCA split the peptide into the component amino acids. Since similar results were obtained when depolymerized TMV protein was used and since end-group studies suggest that TMV subunits consist of single polypeptide chains, the presence of an intramolecular ring closed by a beta-peptide bond is implied. The absence of detectable end-groups in a number of proteins has led to speculation regarding linkages of this kind, but this appears to be the first explicit demonstration. The use of techniques of the kind employed here may help to bring clarification of the nature of the masking in other cases as well.

Ovalbumin.—Ovalbumin, which has no reactive N-terminal residues according to the DNFB method, also fails to react with carboxypeptidase (322, 323). Now, however, Niu & Fraenkel-Conrat have demonstrated by hydrazinolysis that the protein has a C-terminal proline residue and that the C-terminal sequence is .Val.Ser.Pro (63). Akabori *et al.* find that their previous identification of alanine as the C-terminal residue in ovalbumin was in error, and they now confirm the presence of C-terminal proline (113). The failure of carboxypeptidase to attack the native molecule is compatible with the known substrate specificities of this enzyme. The studies of Linderstrøm-Lang, Ottesen and collaborators (324) have shown that subtilisin digestion of ovalbumin splits out Ala.Gly.Val.Asp.Ala.Ala without releasing any other major peptide fragments, phosphate, or carbohydrate. The modified protein remaining after this digestion (plakalbumin II) has a molecular weight differing only minimally from the parent ovalbumin. Steinberg (325) has shown

that subtilisin in low concentrations exposes a previously unavailable C-terminal alanine residue in ovalbumin which, in the presence of carboxypeptidase, can be split off in almost stoichiometric yield together with only traces of other amino acids or peptides. The protein modified by the loss of the single alanine residue, plakalbumin C, resembles plakalbumin II in crystal structure and solubility. Now Ottesen has shown that the initial reaction at pH 8 by which ovalbumin is made susceptible to the attack of carboxypeptidase involves the splitting of a single peptide bond (114). It appears then that subtilisin opens the ovalbumin chain at an alanine carboxyl group and subsequently splits out the hexapeptide without disrupting the continuity of the rest of the molecule.

These studies can be explained in several ways. One possibility is that the N-terminus is masked by carbohydrate or phosphate and that a covalent cross-linkage (disulfide, phosphodiester) keeps the various forms of plakalbumin intact after the splitting out of centrally located amino acids or peptides. Recent studies by Benesch *et al.* are in agreement with earlier work indicating five-SH groups and at least one-S-S- linkage (108). In this case performic acid oxidation of plakalbumin might be expected to yield two fragments. Another possibility is that the N-terminus is masked because of internal cyclization and that the point of subtilisin attack lies somewhere on a closed ring to which is attached a "tail" bearing the C-terminal proline. Attempts in Schramm's laboratory to unmask an N-terminal residue by TCA-treatment, in analogous fashion to the unmasking of the N-terminal proline in TMV, revealed only traces of end-groups by the Edman procedure.

Feather keratin.—Woodin was unable to demonstrate significant amounts of either N-terminal or C-terminal residues in solubilized, performic acid oxidized feather keratin (115, 116). Titration data indicating the absence of free C-terminal carboxyl groups were obtained. Since there is no evidence for the presence of prosthetic groups in the protein, Woodin concludes "that feather keratin is an unbranched cyclic peptide."

Growth hormone.—The presence in bovine growth hormone of two N-terminal residues (alanine and phenylalanine), previously shown by the DNFB method (326) has now been confirmed by Levy & Li using the Edman degradation (117). Treatment of the hormone with carboxypeptidase releases two moles of phenylalanine per mole of protein (327). Because phenylalanine is a preferred substrate for carboxypeptidase, the rapid release of two moles of this amino acid was considered to be compatible either with the presence of two chains each terminating in phenylalanine or with the presence of a peptide chain terminating in the sequence .Phe.Phe. Meyer, Jutisz & Li, using the $LiAlH_4$ reduction method, now find that the protein shows only a single mole of C-terminal phenylalanine per mole, suggesting a branched structure (118).

Taka-amylase.—Ikenaka reports that crystalline taka-amylase, shown previously to have only a single N-terminal alanine residue per 52,500 mol. wt. (328), yields three different C-terminal residues by the hydrazinolysis

method—approximately one mole each of serine, glycine, and alanine (119). The author proposes a three-pronged structure.

In the case of the last three proteins discussed, the cyclic or branched structure is inferred through failure to demonstrate end-groups or from a discrepancy between the number of N-terminal and C-terminal residues. Terminal groups can be missed, and have been in the past, because the method used may not be applicable to all amino acids (incomplete reaction or incomplete recovery; masking by prosthetic groups etc.). For example, Titani, Yoshikawa & Satake, using both the Sanger method and the Edman method, find that horse serum albumin has, in addition to the N-terminal aspartic acid previously reported by several laboratories, an N-terminal half-cystine that has apparently been overlooked (120). If the half-cystine is not merely an "appendage," linked to the rest of the molecule only through a disulfide bridge, a branched-chain or a multiple-chain structure must be considered. In this connection the work of Reichmann & Colvin shows that bovine serum albumin, which on the basis of end-group studies has also been thought to consist of a single chain, can be split into three or four separate chains by performic acid oxidation (121). Two new examples of false negative end-group results in studies using carboxypeptidase have been reported. Halsey & Neurath found native yeast triosephosphate dehydrogenase almost completely resistant to the enzyme, but in the presence of 6 *M* urea (in which carboxypeptidase retained up to 50 per cent of its maximum activity) two moles of C-terminal methionine were released (122). Ramachandran & McConnell were unable to liberate free amino acids from wheat gliadin with carboxypeptidase but obtained qualitative evidence for C-terminal glutamic acid and leucine or isoleucine by chemical end-group methods (123).

For these and other reasons, a positive conclusion about structure cannot be firmly established on the basis of negative results in end-group studies. Explicit demonstration of the cyclic structure, such as that presented by Braunitzer, or explicit demonstration of the branching point is necessary. In this latter connection it is of interest that the case for the presence of gamma-glutamyl bonds in proteins has been strengthened by recent studies of Haurowitz & Horowitz (124).

Relation of Structure to Function

The valuable articles by Porter (329) and by Anfinsen & Redfield (1a) bring together the available evidence which bears on this intriguing problem. Studies from many laboratories have added considerable weight to the active center hypothesis, the hypothesis that there are many elements in the structure of biologically active proteins that are "expendable" (34, 40, 125 to 133). If only short range forces are considered, it is clear a priori that, in some cases at least, only a very small fraction of the enzyme molecule can be directly involved in catalysis. Porter's example of urease (mol. wt. 600,000) and its substrate, urea (mol. wt. 60), is an excellent one. What then is the function of the rest of the enzyme protein?

One possibility is that the configurational specificity of the active center, whether it consists of a series of contiguous residues or a number of widely separated residues held in juxtaposition by the secondary structure of the molecule, is determined by and stabilized by the molecule as a whole. The activity of certain enzymes in concentrated urea solutions does not rule out this possibility unless it can be demonstrated that all of the secondary structure has been disrupted and that the native configuration is not resumed under the conditions of assay (20, 134). The inactivation of many enzymes by denaturation indicates the importance of secondary and tertiary structure in relation to function (135 to 139).

Conversely, the correlation of inactivation with the cleavage of one or more specific bonds or the release of specific fragments cannot be taken to prove the critical nature of these bonds or fragments per se. First it must be established that there are not changes in secondary structure accompanying such cleavages. The problem has been clearly stated and demonstrated by Anfinsen (20). Mild peptic digestion of ribonuclease led specifically to the release of a C-terminal tetrapeptide, apparently with no other peptide bond cleavages. The progressive release of this tetrapeptide correlated well with progressive loss of enzyme activity, but there was, in addition, a simultaneous shift in ultraviolet spectrum which could be attributed to the rupture of a hydrogen bond involving a tyrosine residue. This evidence of modification in secondary structure accompanying the release of the peptide fragment prevents unambiguous correlation of the inactivation with either process alone. Further refinements in protein surgery will be needed.

A second possibility is that the macromolecular structure, at least in some cases, plays an important role as an energy transfer device. The theory of continuous energy bands along polypeptide chains, as originally proposed by Szent-Gyorgi, has been questioned because of the poor conductivity of dry proteins. However, as pointed out by Riehl (140), the electric field in the immediate vicinity of a newly generated ion-pair is extremely high, of the order of 10^5 to 10^6 v./cm., and this high field could bring about a discharge through channels of lower dielectric resistance such as might be provided by a hydrogen-bonded structure. Experimental studies on the conductivity of gelatin at very high field strength are presented which seem to support the theory. Broser & Lautsch described an interesting model experiment demonstrating energy transfer. Poly-DL-(phenylalanine-glutamic acid) was coupled with carbonyl-mesohemin-IX and irradiated with ultraviolet light at a frequency corresponding to the phenylalanine absorption peak. Carbon monoxide was dissociated from the hemin groups with a quantum yield approaching unity (141). The authors interpret their results as indicating a highly efficient energy transfer along the polypeptide chain due to overlapping amplitude functions of neighboring groups in a helix. No evidence of helical structure is presented, however, and the studies of Doty and co-workers actually suggest that in aqueous solution a random structure is probable (142). Shore & Pardee believe resonance transfer to be a more likely mechanism (143). Their

studies on protein-dye conjugates show that energy transmission is proportional to the fluorescence efficiency of the proteins, the latter property being localized almost exclusively in the aromatic side chains (144). The energy absorbed by the aromatic residues is re-emitted with frequencies which overlap those of resonating receiving groups, in this case fluorescent dyes conjugated with the protein. Since the efficiency of transfer in concentrated LiBr and urea solutions was not diminished, it is unlikely that the intact hydrogen-bonded structure is essential. The evidence for resonance transfer is convincing but it is difficult to explain efficiencies close to 100 per cent by this mechanism.

In some cases surprisingly extensive modification fails to alter enzymatic activity. Probably the most dramatic example is found in the preliminary report by Hill & Smith on the degradation of papain (145). Using swine kidney leucine-aminopeptidase they were able to remove up to 120 of the 180 residues of mercuripapain without loss of activity (calculated on a molar basis). The fraction split off represents a calculated average but, assuming all the molecules are degraded to about the same extent, the data indicate that only one third of the papain molecule is essential to its enzymatic activity. If these highly active fragments, averaging only 60 amino acid residues in length, can be purified and characterized, they will represent perhaps the smallest "enzymes" available for further study of the nature of the active center. It will be of interest to see if any of the intermediate partially digested molecules have increased molar activity that might offset loss of activity in more fully degraded molecules.

The studies of Kalnitsky & Anderson (330) on digestion of ribonuclease with carboxypeptidase preparations, interpreted at first in terms of the relation between C-terminal structure and enzymatic activity, have been reinterpreted by Kalnitsky & Rogers (129). The presence of considerable endopeptidase contamination in the carboxypeptidase used leads to the rupture of many internal peptide bonds. Although no well-defined structure-function correlations can be made, the studies show that as much as 15 per cent of the ribonuclease molecule can be lost without apparent loss of enzymatic activity. It should be pointed out that the C-terminal sequence in ribonuclease is now established as .Asp.Ala.Ser.Val (19). Earlier reports of a different sequence are probably attributable to endopeptidase contamination of the carboxypeptidase used or to impurities in the ribonuclease preparations.

Studies by Cole *et al.* (146) confirm and extend the earlier studies by White (331), which show that pepsin preferentially attacks ACTH near the C-terminal end of the molecule and can split off as many as 11 residues without altering biological activity. Further pepsin digestion brought about inactivation that correlated with a split between residues 5 and 6 (.Glu.His.) and aminopeptidase rapidly inactivated by splitting off residues 1 or 2 or both (332). Bell *et al.* (32) now find that the residues beyond 24 can be split off with mild acid hydrolysis without inactivation. None of the smaller fragments examined had activity. Apparently the last 15 of the 39 residues in hog

β-corticotropin are not essential to its biological activity. Any tampering with the N-terminal structure, on the other hand, destroyed activity.

As discussed above, the heptapeptide sequence, 4–10, in ACTH duplicates the heptapeptide sequence, 7–13, in MSH. This sequence is intact in the 28-residue pepsin digestion product, and the MSH activity is likewise intact. It is of particular interest that the enhancement of MSH activity by brief boiling in dilute alkali, noted as early as 1936 by Stehle, is demonstrable with pure β-corticotropin (31). Treatment with alkali, which destroys ACTH activity, is reported to split peptide bonds 1, 2, and 3, confirming their indispensability for ACTH activity and their expendability with respect to MSH activity. The latter is evidently released by the alkali treatment from inhibition imposed by elements of the structure about the common heptapeptide sequence. Dixon, on the basis of the parallel fractionation and parallel destruction of ACTH and MSH activity, also concludes that the latter is a property of ACTH itself (39). The demonstration of definite though feeble MSH activity in chemically pure corticotropin and the isolation of a chemically pure, structurally distinct, but related MSH molecule without ACTH activity appears to resolve a much debated problem in endocrinology (333).

Li *et al.* have shown that hypophyseal growth hormone can undergo extensive digestion by chymotrypsin with no loss in biological activity (125). As much as 30 per cent of the molecule can be converted to fragments soluble in 5 per cent TCA and still the "core" remains fully active.

When, as in these and other hormonal studies, activity must be measured in a complex biological system, alterations in structure occurring after administration must be considered even though the response studied is a rapid one. As discussed above, hypertensin I appears to be degraded to hypertensin II before it acts (45, 46). The converse situation, the addition of amino acid residues to the administered molecule, while it seems less likely, cannot be ruled out a priori.

A survey of studies like those discussed above [cf. Anfinsen & Redfield (1a), Li (1d)] makes it evident that there are some structural features in many enzymes and hormones that appear to be absolutely expendable. The proteins function optimally despite more or less extensive modification. In this context "optimally" is generally equated with "maximally." However, what constitutes optimal behavior for an enzyme functioning in a simple buffered solution may be quite different from what constitutes optimal behavior in the complex milieu of the living cell. In this connection a third possible function for some of the "nonessential" features of enzymes and other biologically active molecules should be considered. Even if only a small part of the molecule is directly responsible for its catalytic role, the rest of the molecule may play an equally important role in orienting the active protein to structural features in the cell and to other enzymes in the cell. The macromolecular structure of an enzyme may be important in determining its thermodynamic activity in the cell under different physiological conditions, its net charge as influenced by interaction with small molecules at sites other

than the active center, or simply its ability to stay inside a semipermeable membrane. Under some circumstances balance in the cell may depend not only on changes in the amount of enzyme present but on changes in specific enzymatic activity. The elements of an enzyme structure outside the active center could represent a "handle" permitting the complexing of the molecule so as to depress or enhance its activity in response to changing metabolic patterns. Swartz, Kaplan & Frech have described examples from their own studies and reviewed several others in which enzymatic activity in crude extracts is masked by the presence of inhibitory substances (147). In the case of DPN pyrophosphatase the inhibitor appears to be a heat-labile protein; in the case of deoxyribonuclease it is a specific ribonucleic acid. These enzyme-inhibitor relations might be very different were it not for the macromolecular nature of the catalytic protein molecules.

Finally, as pointed out by Anfinsen & Redfield, it may be that the "nonessential" features are truly nonessential except as clues to biochemical evolution. Trying to explain their presence in a functional way may be as unfruitful as attempts to "explain" the vermiform appendix.

Sedimentation, Diffusion and Viscosity

Significant progress has been made in the practical application of the theory of pre-equilibrium distribution of a sedimenting material in the ultracentrifuge cell (334). This method permits determination of the sedimentation and diffusion constants, and thus of the molecular weight, from data obtained in a single short centrifuge run. Klainer & Kegeles have described a new procedure employing the usual schlieren optical system of the ultracentrifuge equipped with a phase plate (148). Their method was tested on bovine serum albumin and ribonuclease and gave results in excellent agreement with those found by other methods (149). The Archibald principle was also applied by Reichmann & Colvin in determining the molecular weight and homogeneity of performic acid-oxidized bovine serum albumin (121) and globin (150) and by Brown, *et al.* in an investigation of β-corticotropin (33). In the case of ribonuclease and β-corticotropin it becomes possible to compare the physiochemical data with the minimum formula weights determined by quantitative amino acid analysis and, in the latter, from the structural formula. The chemical methods yielded values of 13,895 and 4567 respectively; the ultracentrifugal data gave values of $14{,}000 \pm 200$ and 4720.

A different application of this theory was described by Yphantis & Waugh, who devised a special cell permitting separation of the liquid column into two superimposed layers during deceleration (151, 152). Analysis of the two fractions provides the data for determining both the sedimentation and the diffusion constants and thus the molecular weight of a substance. Since biological activity can be used as a measure of concentration, the method is particularly suitable for determining the molecular characteristics of active substances at very low concentration and even in an impure state. A study of the heparin cofactor by this method showed that the active principle sedi-

ments at a different rate than the single peak seen in the schlieren pattern (153). All these methods have the important additional advantage of being applicable to low molecular weight substances. Studies on raffinose (148), vitamin B_{12}, and DPN (152) again gave results in remarkably good agreement with the chemical molecular weights.

The synthetic boundary cell, as shown by Hersh & Schachman (154), can be used to study the sedimentation of a boundary produced between two different concentrations of the same solute. In some cases this permits the calculation of the sedimentation constant extrapolated to zero concentration from the data obtained in a single run.

The determination of partial specific volume by ultracentrifugation in mixed solvents of varying densities was discussed by Katz (155). He showed on theoretical grounds that a higher partial specific volume than that of the anhydrous protein can arise solely from entropy effects attributable to the relative sizes of the molecules, invalidating to some extent the conclusions regarding hydration of proteins reached by this method. Martin, Cook & Winkler called attention to the molecular weight increase caused by exchange of labile hydrogens for deuterium when heavy water is used with this method (156).

The effects of hydrostatic pressure (157), concentration dependence of the sedimentation constant (158), and rapid reversible equilibrium between sedimenting components (159) have also been considered from a theoretical point of view. Trautman described procedures which facilitate the analysis of schlieren patterns (160).

Molecular weights based either on sedimentation and diffusion studies, osmotic pressure measurements, or light scattering data have been reported for the following proteins: β-corticotropin (33); histones (162); several cytochromes (163, 164, 167); ribonuclease (149); myoglobin (165); elastase (166); penicillinase (161); ovalbumin (168); malic dehydrogenase (169); isocitric enzyme (170); hemoglobin (171), tetanus toxin (172); serum albumin (173, 174, 179); phosphoglucomutase (175); conalbumin (176, 177); heat stable muscle protein (178); capsular polyglutamic acid (179); hyaluronidase inhibitor (180); peptomyosin B (181); glyceraldehyde-3-phosphate dehydrogenase (182, 183); arginase (184); aldolase (182); xanthine oxidase (185); collagen (186); lipovitellin (187); clover leaf protein (188), and ribonucleoprotein (189).

Acid-Base Equilibria

Simple electrostatic theory, which has been successfully used in describing acid-base equilibria in proteins, is limited to spherical models impenetrable to ions, with the charges located on the surface. Recently, however, attempts have been made to extend it to models which permit partial or full penetration of the sphere by ions, charges being localized either on the surface or in the interior of the molecule (190, 191). In the latter case, as pointed out by Hill, the low dielectric constant of the interior increases the electro-

static effect considerably (192). Empirical determination of the electrostatic factor from titration curves and its comparison with those calculated for various models, and especially the variation of the electrostatic factor with ionic strength, permit conclusions regarding molecular structure. Results on ribonuclease were compatible with a compact, impenetrable, spherical model, with possibly a slight degree of asymmetry (193, 194). There were no signs of deformation in the pH interval from 2 to 12. This conclusion was verified by intrinsic viscosity determinations in the same pH range (195). Serious deviations were encountered, however, with bovine serum albumin (196). In the pH range 4.3 to 10.0 it showed a normal behavior, the electrostatic factor corresponding to that for an ion-impenetrable sphere, with the generally accepted dimensions. Below this pH range, however, the data could be accommodated only by assuming a sharp decrease in the electrostatic factor. Tanford believes this to be the expression of an expansion of the molecule. In line with this interpretation, intrinsic viscosity determinations showed that the molecule is unchanged between pH 4.3 and 10.0 but undergoes considerable expansion outside of this range (197, 198, 199). That these viscosity changes do indeed reflect changes in molecular shape is shown by the sedimentation studies of Harrington, Johnson & Ottewill (173). However, as pointed out by Foster & Sterman (200), the change in the electrostatic factor occurs between pH 4.3 and 4.0, whereas the expansion which is slight in this range, becomes significant only below pH 4.0. They believe that the increased binding of hydrogen ions is the cause and not the consequence of the increase in molecular volume. The same conclusion was drawn from the effect of urea on intrinsic viscosity, optical rotation, and the binding of hydrogen ions by serum albumin. According to this picture the carboxyl groups are involved in strong ionic or hydrogen bonds with cationic groups, and the rupture of these by binding of hydrogen ions or urea causes the anomalous titration curve, and simultaneously transforms the molecule into an expendable form. The two forms, native and expandable, exhibit different electrophoretic mobilities; apparently the equilibrium between them is established slowly, so that they separate during electrophoresis and their relative proportion can be estimated (78). The equilibrium is third order with respect to hydrogen ions. Acetate ions seem to favor this "isomerization" (80). That this phenomenon is not caused by heterogeneity is shown by the fact that at pH 3.42 the transformation is complete and, again, only a single component is present. On the other hand, fractionation of rat serum albumin with Zn-ethanol yielded a preparation which was homogeneous even at low pH (201). These two results are apparently contradictory, but it should be pointed out that they were obtained with proteins from different species. The isomerization of bovine serum albumin should be differentiated from the still slower aggregation phenomenon which occurs at low pH values. This, as shown by Kronman, Stern & Timasheff, is dependent on an ionic impurity, deionized albumin being much less prone to aggregation than the ordinary preparation (202).

Expansion of the protein molecule at low pH seems to be quite a general phenomenon. Conalbumin below pH 4 also shows a reversible, isodimensional swelling, which particularly at higher ionic strengths is complicated by aggregation phenomena (176). The calculations of Hill show than an $\alpha \rightarrow \beta$ transformation may be a reasonable explanation for the swelling of the protein molecules caused by the excessive intramolecular electrostatic repulsion (203).

Spectrophotometric methods make it possible to follow unequivocally the dissociation of tyrosine residues in proteins, the usual difficulties in assigning a certain portion of the hydrogen ion dissociation curve to a particular group being eliminated by the specificity of the method. Tanford, in order to explain the anomalous titration of tyrosine groups in bovine serum albumin, assumed that they were hydrogen bonded to carboxylate. This assumption, however, was later rejected by him on thermodynamic grounds and because of the disproportion between the number of tyrosine and COOH groups (196). The hydrogen bonding should lead to two different classes of carboxyl groups which are not apparent in the titration curves. Most likely the carboxylate ions are involved in electrostatic bonds and the tyrosine residues in H-bonds with some as yet unidentified groups, possibly imidazole groups. However, in insulin, Laskowski *et al.*, using differential spectrophotometric titration, were able to demonstrate the existence of a tyrosine, hydrogen bonded to a carboxylate ion (204). In ribonuclease half of the tyrosine residues are unable to ionize in the native molecule (193) but this effect is too strong to be attributed simply to hydrogen bonds or salt bridges. Tanford assumes that these tyrosine groups are buried in a hydrophobic cluster, in the sense of the "hydrophobic bond" of Kauzmann.

An extensive theoretical treatment of the potential hydrogen bonded structures in proteins and their effects on acid-base equilibria has been given by Laskowski & Scheraga (205, 206).

Theoretical treatment of nonspherical models is difficult, although substantial progress has been made in this direction by Hill (192). In all the theories mentioned so far the charges are assumed to be uniformly distributed and far enough from each other to make interaction between neighboring groups negligible. One would think that this is far from the real situation in proteins, but the remarkable success of the simple theory, with reasonable pK values, indicates the soundness of the assumptions in many cases. Neighboring groups can affect each other's dissociation to a great extent, a fact long recognized and emphasized recently by Hill (207). He presents a mathematical treatment of the forces arising between matching constellations of charged groups in which the fluctuating charges may be frozen because of mutual interaction.

Light scattering studies by Timasheff *et al.* (208) on isoionic, salt-free, bovine serum albumin showed the reciprocal reduced scattering intensity to be proportional to the square root of the protein concentration, as predicted by the Kirkwood-Shumaker theory. The magnitude of the fluctuating

charge determined from the light-scattering data agreed well with that calculated from the titration curve. The former was determined also for conalbumin from the titration curve of the salt-free protein (209). The fluctuating charge is small compared to the net charge the protein can assume outside the isoelectric region and it is very effectively screened by relatively low salt concentrations. Its importance, therefore, is confined largely to the isoionic salt-free protein. A strong concentration dependence of the diffusion constant of salt-free isoionic serum albumin may also be related to interactions of this kind (210).

Interaction with Small Molecules and Ions

The structural basis for the binding of small molecules and ions to proteins is poorly understood. It is clear, however, that in most cases a favorable constellation of at least two side chains of the protein is involved, since the binding energies are much higher than those observed with any isolated amino acid. Furthermore, many proteins which certainly have free side groups do not bind ions or small molecules to any extent. Ultimately, an understanding of the binding phenomena will probably depend on a mapping of the surface of the protein molecule. Peticolas & Klotz have shown by varying the distance between a tertiary amino and a carboxyl group of an azo dye that the binding to human serum albumin takes place through both these groups to surface binding sites which are apparently 12 Å apart. Increase or decrease of the distance between the two groups on the dye resulted in suppression of the binding to one of the sites. Bovine serum albumin bound the dye only through one of the groups, probably the carboxyl, irrespective of the distance between them (211).

Extensive data have been obtained on the binding of metals (73, 212 to 221), vitamin B_{12} (222, 223) and steroids (224, 225, 226) to various enzymes and proteins.

The binding to protein of ions with large hydrophobic parts may result in profound changes in molecular structure in addition to the expected effect on net charge. Binding of sodium dodecylsulfate is the classical example of this phenomenon. β-Lactoglobulin binds 2.5 moles of *n*-octylbenzene-*p*-sulfonate normally, but further binding is accompanied by a sudden increase in binding capacity, together with the appearance of a new electrophoretic component. Hill & Briggs interpreted this as an opening of the molecule, the first bound anions serving as a "molecular wedge," leading to the exposure of new sites and to changes in electrophoretic properties (81). Probably similar changes are responsible for the splitting of bovine serum albumin into two components in acidic acetate buffers (80). A fragmentation of legumin into pieces with one-sixth the original molecular weight in the presence of sodium dodecylsulfate was observed by Brand & Johnson (227), and a disaggregation of a soluble keratin was described by O'Donnell & Woods under similar conditions (228). Binding of large anions, which have no tendency for micelle formation, results in neutralization of the protein acid to the isoelectric point,

with subsequent precipitation. Studies have been performed on the interaction of human serum albumin with perfluoro-octanoic acid (229) and of plasma proteins with tungstic and picric acids (230), with special reference to the precipitation phenomena. No selective precipitation of any particular protein was apparent in the latter studies. On the other hand, Rivanol, a cationic dye, is capable at neutral pH of precipitating all the plasma proteins except γ-globulin, which is understandable in view of the high isoelectric point of the latter (231).

TMV binds approximately one mole of safranine O per protein subunit and the number of binding sites is doubled when the C-terminal threonine residues are removed by carboxypeptidase (232). The importance of a specific configuration of the binding site is shown by the preferential adsorption on wool of one of the optical antipodes of mandelic acid or its derivatives (233). Studies of the interaction of a divalent hapten with its specific antibody by light scattering methods were compatible with the assumption that all hapten-antibody bonds are equivalent and that there are two binding sites on the antibody molecule (234). The reaction is reversible and from the equilibrium constant and its dependence on temperature the thermodynamic constants of the reaction were determined.

Protein-Protein Interaction and Interaction with Other Large Molecules

The same fundamental kinds of forces are involved in interactions with larger molecules but here a strong interaction is possible because of the cooperation of a large number of weak bonds. The interaction can be through long-range forces, provided by the net charge of the particles, or through more specific bonds involving side chains. In this respect the intermolecular and intramolecular side chain interactions will be of the same kind and a stronger interaction will be expected with proteins having a large number of free side chains than with those in which the side chains are involved in internal stabilization of the molecule. Obviously, the side chain interactions can be very specific, inasmuch as the protein surfaces facing each other may have a complementary pattern which permits reaction among a large number of groups. As already mentioned, such a specific pattern may also be made possible by the freezing of fluctuating charges into a favorable configuration, the bonds in this case being of an electrostatic nature (335). The interaction of molecules may lead to an orderly arrangement which may, on the one hand, be limited to a few molecules or may, on the other hand, extend to an indefinite number of molecules. The first situation is generally described as association, the second as polymerization. Both reactions are generally reversible, the equilibria being governed by association constants in the classical sense. If the polymerization is end-to-end, long chains are formed which by branching can build up a three dimensional network leading to gelation. On the other hand, the interaction may result in a random grouping of the molecules in a soluble aggregate or a precipitate. One of the major factors

limiting aggregation is the mutual electrostatic repulsion of the molecules but this can be overcome by strong side-chain interactions. If only the net charge were operative, the solubility curve would be symmetrical about the isoelectric point. Actually some proteins show increase of solubility with increase of net charge above the isoelectric point but decrease of solubility below the isoelectric point (e.g. fibrinogen, myosin). The decrease in solubility below the isoelectric point shows that strongly interacting sites are exposed or generated at a low pH. Similarly, denaturation, while it leaves the net charge practically unaffected, eventually results in aggregation and precipitation by exposure of a great number of side chains. Primarily electrostatic interaction and precipitation will result on the mixing of proteins or polyelectrolytes of opposite charge or both, and many interactions of this type have been investigated (235 to 239).

Turbidimetric studies by Coleman & Edelhoch on the interaction of nucleic acids with proteins show that ribonuclease binds both ribonucleic and deoxyribonucleic acids but that the binding of the former is much stronger, correlating with the specificity of the enzyme (240). Cytochrome-*c*, on the other hand, a protein of the same size and isoelectric point as ribonuclease binds deoxyribonucleic acid much more strongly than ribonucleic acid, supporting the specificity of the interaction with substrate.

Several proteins show a great tendency for dimerization or polymerization. Since in this case the protein-protein interaction involves identical molecules, net charge effects must be of little importance and specific bonds or fixed charge patterns must be assumed. According to Welsh, tobacco mosaic virus shows a slow end-to-end dimerization and the reaction involves the uptake of 3300 hydrogen ions per virus particle (241). The dimerization could be brought about by addition of methylene blue and the number of dye molecules bound was equal to the number of hydrogen ions necessary for the spontaneous dimerization. Why so many ions are involved in an end-to-end polymerization is a question which remains to be answered. O'Konski & Haltner determined the rotatory relaxation time of the virus by measuring the transient electric birefringence of the system (242). Some solutions contained only the monomer, others the dimer as well. The proportion of these was a property of the individual preparation and did not seem to be a function of the protein concentration, as would be expected in the case of an equilibrium reaction, nor did it vary with the age of the preparation, a result in contradiction with the aforementioned experiments. Spontaneous dimerization of tetanus toxin with loss of toxicity (spontaneous toxoid) was also described (172).

Myosin, even when free of actin, shows a pronounced tendency for aggregation. The initial, reversible phase of this process is strongly temperature dependent with a negative temperature coefficient, as shown first by Laki & Carroll (243) and confirmed by Spicer & Weise (244). The irreversible phase has a positive temperature coefficient and according to the light scattering measurements of Holtzer consists of side-to-side association (245). On the

other hand, flow-birefringence studies show that, depending upon pH and ionic strength, considerable lengthwise association may also occur, even in fresh myosin solutions (246). These results probably explain the widely divergent results of different authors on the size and shape of the myosin molecule. Myosin forms complexes readily with actin and the synthetic actomyosin thus obtained strongly resembles the substance extracted from muscle at high ionic strengths and prolonged extraction, the natural actomyosin or myosin B. Some doubt exists, however, as to the identity of the two substances. It has never been possible to demonstrate unequivocally the presence of actin in myosin B preparations. Weber's recent experiments show quite convincingly the presence of myosin in the supernatant fluid when myosin B is centrifuged in the presence of ATP (247). Substantial amounts of actin could not be recovered from the pellet. Gergely's light scattering experiments, on the other hand, demonstrate an identical response of both actomyosins toward ATP (248). Bárány and co-workers, investigating the mechanism of the polymerization of actin, find that only half of the —SH groups of actin react with Salyrgan in the polymerized state, whereas all react in the monomeric form (249, 250). From this it is concluded that half of the —SH groups are involved in intermolecular bond formation. They also demonstrated the participation of amino groups in this process and postulated the formation of hydrogen bonds between the —SH and amino groups as the mechanism of polymerization. Depolymerization could be effected by blocking reagents for these groups and by reagents which rupture hydrogen bonds. ATP exerted a protective action against all these depolymerizing agents (251).

Secondary and Tertiary Structure

Simple polypeptide chains.—According to the thermodynamic considerations of Schellman the stability of a helix depends on the free energy change of the unfolding, and this in turn is a function of the length of the helix and the strength of the stabilizing hydrogen bonds (252, 253). The theory predicts that helices below a certain length will be unstable and will show increasing stability as the length increases. In solvents capable of hydrogen bond formation the work of uncoiling the helix will be less, because there will be exchange of hydrogen bonds within the chain for hydrogen bonds with solvent molecules. In solvents with poor hydrogen bonding ability, the uncoiling will require a higher energy expenditure. In weak hydrogen bonding solvents, therefore, the limit of stability will be at shorter helix lengths than in strong hydrogen bonding solvents. The beautiful work of Doty and co-workers and Blout and co-workers on synthetic polypeptides fully confirms these predictions. Poly-γ-benzyl-L-glutamate molecules of known chain length were prepared by controlled polymerization (254). Infrared absorption data showed that in the solid state chains below approximately 128 residues were predominantly in the stretched β-form, whereas longer ones were in the folded α-form (255). The polypeptide was found to be in an α-helical configuration in solvents of poor or no hydrogen bonding ability and in the form of a random

coil in solvents capable of hydrogen bonding (142). Data on optical rotation, viscosity, and double refraction of flow showed the sudden transition from the helical configuration to the random coil as the proportion of hydrogen bonding component in the solvent was increased (256). Side-chain interactions can obviously stabilize or labilize the helix to a great extent. Poly-α-L-glutamic acid is in the helical form, but sodium polyglutamate is completely stretched due to the strong electrostatic repulsion between the negative γ-carboxylate groups (257). Water, with its very high hydrogen bonding ability, would be expected to be an unfavorable solvent for helix formation and even very long chains may be unable to maintain a helical configuration in this solvent. The work of Harrington & Schellman, indeed, shows that oxidized ribonuclease, although it is a single polypeptide chain of 124 amino acid residues, is in the form of a random coil in water (258). Evidently, favorable side-chain interactions, disulfide bridges or other cross-linkages are necessary for the maintenance of the helical configuration in water. Hvidt & Linderstrøm-Lang's deuterium exchange experiments show that in insulin and in ribonuclease one group of hydrogen atoms attached to nitrogen or oxygen is rapidly exchangeable, whereas another group is only very slowly exchangeable (259, 260, 261). They believe that the slowly exchanging atoms belong to the areas of the molecule stabilized by a helical configuration. In the case of insulin the number of slowly exchangeable hydrogen atoms corresponds to the number within the segments stabilized by disulfide cross-linkage. On the other hand, the isolated A-chain of insulin and some di-, tri-, and tetrapeptides, lacking a stable secondary structure, have only freely exchangeable nitrogen- or oxygen-bound hydrogen atoms. Infrared spectroscopic studies provide direct evidence that the less readily exchangeable hydrogen atoms are situated in the peptide backbone (262).

Optical rotation is a useful index of the state of the protein molecule and also provides a simple method for following the kinetics of changes in structure (197, 263). Until recently, however, it has been of little value in providing information on the structure itself. A fully random polypeptide chain exhibits an optical rotation close to the sum of the individual contributions of its asymmetric carbon atoms (264). Fitts & Kirkwood have pointed out that when the chain assumes a helical configuration an additional contribution arises from the fact that the helix itself has no reflection symmetry and thus is optically active even in the absence of asymmetric carbon atoms (265). When only the asymmetric carbon atoms are operative, the wavelength dependence of the optical rotation (rotatory dispersion) conforms to the single-term Drude equation. The same situation occurs when the helical structures are short or when the effects due to the helical configurations are internally compensated. The native globular proteins that have been investigated (173, 263) conform to the Drude equation, probably not because of the complete absence of helical structures, but because of the shortness of the helices and because of internal compensation. Shortness of the helical segments, a

result of the high proline and oxyproline content, may be responsible for a similar observation on gelatin by Cohen (266). Deviation from the single-term Drude equation may be interpreted as the result of helical configuration. Moffitt has derived an equation describing the rotatory dispersion of an infinitely long helix, which accounts for the deviation from the single-term equation (267, 268, 269). Experimental data on polyglutamic and polybenzylglutamic acid are in excellent agreement with the theory (268). If further data substantiate the theory, rotatory dispersion will be a valuable tool in the investigation of the secondary structure of proteins. Fitts & Kirkwood (265, 270) presented a theory of the optical rotatory power of helical molecules and the rotation predicted for polybenzylglutamate was in good agreement with that found experimentally by Doty & Yang (256).

Multiple-chain proteins.—Multiple chains in a complex protein may be held together by covalent bonds (disulfide or phosphodiester linkages), thus constituting a true molecule in the chemical sense; or the chains may be linked only by secondary forces. One can visualize two situations in the latter case: (*a*) the chains may be interlocked intimately so that separation is not possible without disruption of secondary and tertiary structure, that is, without denaturing the protein; (*b*) they may represent compact subunits held together only by bonds weaker than those within the units, making possible a dissociation without denaturation. Structures of the second kind are closer in a chemical sense to aggregates than to molecules, but for many purposes they can be dealt with as molecules because of their high degree of organization, and their specific and uniform size and shape, properties which clearly differentiate them from random aggregates. The term structural subunit, which has been used rather loosely, should probably be reserved for covalently bonded structures joined to one another only by secondary forces. The products of proteolytic degradation, for example, should not properly be called subunits, even though the fragments appear to be few in number and well characterized. The appearance of large intermediates during breakdown may only reflect the specificity of the proteolytic enzyme, some peptide bonds being split at a much higher rate than the rest. It does not necessarily imply that such "subunits," as they exist in the protein molecule, have any unique structural or functional significance.

Dissociation of protein molecules into subunits can be achieved by several types of agents: (*a*) hydrogen bond breaking substances (urea, guanidine HCl); (*b*) detergents (sodium dodecylsulfate); (*c*) extreme pH values; and (*d*) high ionic strengths which may be associated with special ion effects (LiBr). Reichmann & Colvin (150) showed that globin prepared from horse hemoglobin is split into quarters (mol. wt. 16,000) by lowering the pH to 2.0. Horse hemoglobin did not reach but approached this stage of dissociation at this pH, the average molecular weight by osmotic pressure being approximately 20,000. Human carbonylhemoglobin, on the other hand, dissociated into halves when the pH was lowered to 3.5 (336). Human hemoglobin is

apparently more resistant to dissociating effects (see also below), or possibly the investigations with the human material were not extended to sufficiently low pH values. The fact that hydrogen ions alone are sufficient to dissociate the molecule and performic acid oxidation is without any effect is understandable in view of the absence of disulfide bridges in the molecule. Gutter and co-workers found only a slight decrease in the molecular weight of horse carbonylhemoglobin in 6 *M* urea and no change at all with human carbonylhemoglobin (171). Mercaptoethanol alone, and to some extent ethanolamine, also slowly decreased the molecular weight of the protein. The mode of action of these two agents is obscure. Most effective was the combination of 6 *M* urea with 1.4 *M* mercaptoethanol, but even so the stage of half molecules was not reached. Perhaps the results can be explained by the stabilizing effect of the heme groups, or the tendency of some proteins to aggregate in urea solutions even at neutral pH. It may be relevant in this respect that McKenzie, Smith & Wake (168) found a pronounced aggregation of ovalbumin in 7 *M* urea at pH 6.2, aggregation which was not prevented by cysteine or *p*-chloromercuribenzoate, and so does not appear to be attributable to a disulfide interchange. Bovine serum albumin, on the other hand, showed aggregation only at high pH and this was prevented by sulfhydryl reagents.

G. Weber (337), on the basis of depolarization of fluorescence measurements, concluded that bovine serum albumin dissociates into halves in acidic and alkaline solutions. Harrington, Johnson & Ottewill, while confirming the increase in rotational relaxation time of the molecules at low pH, could find no evidence for dissociation from their sedimentation and diffusion measurements (173). These contradictory interpretations may be resolved by assuming a dumbbell model for the protein. Two large segments of the molecule linked by a short connecting segment may be immobilized by secondary forces at neutral pH where the molecule is compact and fairly symmetrical. At low pH, when the molecule expands and assumes an elongated shape, these two subdivisions could acquire rotational freedom around the common axis and the relaxation time would approach a value characteristic of the size of the individual "subunits."

The complex nature of myosin is well recognized. Attempts to disintegrate it have been conducted along two lines: (*a*) by physical or physicochemical agents; (*b*) by proteolytic degradation. The second approach, as discussed above, gives results which are difficult to interpret in terms of structural or functional subunits. Unequal labeling of the meromyosins, fragments of myosin obtained by trypsin digestion, demonstrates that they may at least be subunits in biosynthesis (271, 272). L-Meromyosin disintegrates further in the presence of urea into fragments of about 9000 mol. wt., the protomyosins (273). The peculiar dependence of the process on pH, urea concentration, and temperature, and the apparent second order kinetics deserve further investigation. The fact that the protomyosins cannot be obtained from native myosin, but only from L-meromyosin, which has already undergone proteolytic degradation, makes their standing as subunits ques-

tionable. Locker (274) investigated in detail the dissociation of myosin by heat coagulation, a method used previously by Engelhardt to separate the adenylic deaminase activity from myosin proper. After heat coagulation of the bulk of the protein at 53° some 8 to 10 per cent remained in the supernatant. This material included a myosin-like fraction which contained the enzymic activity and a low molecular weight, water-soluble fraction showing three components in the ultracentrifuge, the main peak having a molecular weight of 20,000 to 30,000. All these substances, including the parent myosin, had a C-terminal isoleucine, but in the fragments its liberation was rapid, whereas in myosin it was very slow. Whether the end group of myosin arises from the low molecular weight component or not could not be decided.

Extensive physicochemical studies by Boedtker & Doty (186) on a soluble collagen extracted by mild procedures from carp swim bladders by the method of Gallop (338) show that this protein is homogeneous. The material shows an unusual heat lability, dissociating into three polypeptide chains in 30 min. at 34° C. The finding of three constituent chains is in agreement with the model proposed for collagen by Rich & Crick (275).

Urea, as well as high pH or low ionic strength, dissociates the peanut protein, arachin, into fragments of approximately 22,000 molecular weight, less than one-tenth that of the native molecule. Johnson & Naismith (276) demonstrated that the process is slow and occurs in several steps. First there is a rapid, slight swelling of the molecule which is reversible, then the molecules are slowly transformed into a highly viscous, slowly sedimenting material which is insoluble in the absence of urea. Finally, a slowly sedimenting material appears which has a low viscosity and is soluble in the absence of urea, corresponding to the afore-mentioned subunits. Interestingly enough, in guanidinium chloride only the highly viscous product, corresponding to the extended molecule, is obtained and the fragmentation into soluble products does not take place. Another plant protein, legumin, is dissociated into subunits by sodium dodecylsulfate (227). As the number of bound detergent ions is increased, there is at first an aggregation, apparent in the ultracentrifuge, which leads to precipitation when about 80 detergent ions are bound per molecule. The precipitate redissolves slowly at room temperature, more rapidly at 45° C., or on the addition of more sodium dodecylsulfate. At the same time the aggregated polydisperse material is replaced by a more homogeneous, more slowly sedimenting material which, according to the sedimentation and diffusion data, appears to have approximately one-sixth the molecular weight of the original legumin.

Interesting results have been obtained using nonaqueous solvents, which by virtue of their hydrogen bonding ability, low dielectric constant, or some other unknown mechanism, are able to dissolve many proteins. This approach may be especially useful for the study of fibrous proteins, insoluble in aqueous solvents, and also for proteins showing a great tendency for association in water (277). Of the 15 solvents investigated by Rees & Singer, hydrazine and ethylenediamine proved to be the most effective (278). Most of

the proteins in these solvents showed a single peak in the ultracentrifuge. Bovine serum albumin, however, sedimented with two peaks, possibly because of an equilibrium between two different configurations, a situation also encountered in water at low pH (78). Insulin in N,N-dimethylformamide gave a molecular weight of 6200 by osmotic pressure measurements, a value now well established as the weight of the fundamental unit. From the variation of the molecular weight with concentration, the association constant for the dimerization was calculated and from this a free energy change of −4.4 kcal./mole was estimated for the process (279). The corrected sedimentation constants in the nonaqueous solvents were markedly lower in most cases than the corresponding ones in aqueous solution, indicating an unfolding of the molecule. Strong anhydrous acids are also good solvents for many proteins (280). Bovine serum albumin sedimented as a sharp, symmetrical boundary in trifluoroacetic acid. Preliminary light-scattering measurements with some proteins gave the following molecular weights in this solvent: silk fibroin 60,000, rat tail collagen 70,000, and insulin 6000. Ribonuclease and lysozyme could be recovered from solution in strong anhydrous acids (HF, $CF_3 \cdot COOH$, HCOOH) in a water-soluble and fully active state (281). Longer treatment, or higher temperature, caused a drop of 15 to 20 per cent in the sedimentation constant of the recovered material and a concomitant loss of activity. These changes were correlated with the opening of peptide bonds. Insulin is also soluble in anhydrous liquid ammonia and can be recovered from this solvent in fully active form with unchanged physicochemical properties (282). The recovered insulin contains some 30 moles of water per 6000 mol. wt. These must be so tightly bound to the protein that they do not react with the ammonia.

Many of the mechanical properties of high polymeric structures can be explained by assuming an alternation of amorphous regions, where the chains are randomly coiled, with crystalline portions where they are arranged parallel to each other. Flory (283) has applied this model to fibrous protein molecules like collagen, elastin, and myosin with considerable success. According to this representation the thermal shrinkage of the fibers is caused by a melting of the crystallites and accordingly has all the characteristics of a phase transition. The elastic behavior of the fibers can also be explained on the basis of the above model (284). The "melting point" of collagen can be determined accurately with a dilatometer (285). Decreasing the volume fraction of the protein by bringing about swelling in glycol vapor depresses the melting point and from this effect the heat and entropy change associated with the fusion of the crystallites can be calculated. These are in fair accord with the assumption of the breaking of one hydrogen bond per residue during the melting.

Denaturation

Denaturation in the following discussion is defined as a change in secondary and tertiary structure without modification of covalently bonded struc-

ture. From studies of denaturation useful information often may be obtained about the native structure. With some proteins the covalent side-chain interactions are sufficient to prevent complete randomization of the chain, and a refolding into the native structure or a closely related structure when the denaturing agent is removed is more likely. In this case the denaturation is reversible. In the absence of such bonds refolding is not possible and the denaturation is irreversible. The unfolding of the peptide fabric frequently results in the exposure of previously hidden reactive groups, and also in the disappearance of activity when the latter is dependent on the secondary structure. Unmasking of reactive—SH groups is probably the most commonly studied example. γ-Globulins were shown to be devoid of reactive—SH groups in the native state but 10 to 17 were exposed on heat denaturation (286).

Denaturation may change the ionization of side chains through a general modification of the electrostatic field brought about by changes in configuration or more specifically by rupture of hydrogen or salt bridges. The result is a pH change and an apparent liberation of acid- and base-binding groups (135, 287). Growth hormone, outside its stability zone (pH 4 to 11.5), was transformed into a component characterized by a higher electrophoretic mobility. Whether this was due to exposure of new charged groups, shape changes, or both, was not determined (288). Kinetic experiments on the urea denaturation of ovalbumin in heavy water revealed a threefold decrease in rate as compared with that in water, a finding explained by the energy difference between the deuterium bond and the hydrogen bond (289).

Some substances may stabilize the protein to a great extent, possibly by forming new bridges between the side chains. For example, bovine serum albumin is stabilized against thermal denaturation and tryptic digestion by Ca^{++} and Mg^{++} (290). Prostatic acid phosphatase is stabilized against surface denaturation by proteins, polypeptides, and, particularly, long-chain polyamines. Amino groups attached to a long chain seem to be essential for this protective action (291).

The thermodynamic data for the activation process were determined for the denaturation of a series of viruses with hemagglutinin activity (292) and for isohemagglutinin β (A_1O) (293). Thermal denaturation of TMV converts the rod-shaped particles into spheres. The rate of this process was determined at several temperatures by electron-microscopic observation and the activation energy calculated from these data was in good agreement with that determined by conventional methods (294).

Heated ovomucoid apparently loses its antitryptic activity and its ability to form a precipitate with its specific antibody, without any appreciable change in viscosity or in electrophoretic and sedimenting properties (295).

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AMINO ACID AND PROTEIN METABOLISM[1]

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AMMONIA

The central metabolic position of ammonia has received renewed attention in recent years. To the list of known ammonia-producing reactions in mammals, summarized by Bessman (1), has been added the following: formylation of THFA[2] from formiminoglutamate (2, 3) and, in microbial sources, L-alanine dehydrogenase (4); formylation from formimino glycine (5 to 11); reductive deamination from glycine (12); and the degradation of glucosamine-6-phosphate (13). Since the fate of the nitrogen of numerous compounds is at present unknown, this list may be expected to expand annually. Crystalline glutamic dehydrogenase isolated from corn leaves by Bulen (14) is DPN-specific, sensitive to sulfide reagents, and in general resembles the enzyme obtained from mammalian sources. Since the amino nitrogen of L-amino acids can be directed toward glutamate by transamination, it appears that glutamic dehydrogenase is the enzyme responsible for the major production of ammonia in hepatic metabolism in the steady state; it is not equally certain, however, that glutamic dehydrogenase universally occupies so central a role. Of various strains of *B. subtilis* which have been isolated, only those which possess glutamic dehydrogenase can use NH_3 as the sole nitrogen source (15). However, a mutant (S—) which can use ammonia lacks glutamic dehydrogenase yet is capable of oxidizing glutamate (4). Fractionation of lysozyme-treated cells yielded a DPN-specific L-alanine

[1] The survey of the literature pertaining to this review included journals received through November, 1956.

[2] The following abbreviations are used in this chapter: AICAR for 4-amino 5-imidazole carboxamide ribotide; ADP for adenosinediphosphate; AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; CSA for cysteine sulfinic acid; CTP for cytidinetriphosphate; DAP for diaminopimelic acid; DNA for deoxyribonucleic acid; DNP for 2,4-dinitrophenol; DOPA for 3,4-dihydroxyphenylalanine; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); EDTA for ethylenediamine tetraacetic acid; FAD for flavin adenine dinucleotide; FIG for formimino glycine; FGAR for formyl glycinamide ribotide; FMN for riboflavin phosphate; GAR for glycinamide ribotide; GDP for guanosinediphosphate; GMP for guanosinemonophosphate; GTP for guanosinetriphosphate; IDP for inosine diphosphate; IGP for imidazole glycerol phosphate; IMP for inosinemonophosphate; INH for isonicotinylhydrazide; ITP for inosinetriphosphate; PCMB for *p*-chloromercuribenzoate; P_i for inorganic phosphate; PB_i for inorganic pyrophosphate; PRPP for 5-phosphoribosylpyrophosphate; RNA for ribonucleic acid; THFA for tetrahydrofolic acid; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form); Tris for trishydroxymethylaminomethane; and UTP for uridinetriphosphate.

dehydrogenase; the reaction is reversible, thereby permitting the organism to grow on ammonia and accomplish glutamate oxidation by transamination from glutamate to pyruvate with subsequent oxidation.

Although the role of a general L-amino acid oxidase has been minimized there continue to be reports of such enzymes. The most recent additions are the L-amino acid oxidase of a mollusc, *Mytilus edulis* (16), and of turkey liver (17). The latter is unusually active toward basic amino acids yielding the α-keto analogues of arginine, ornithine, and lysine, each of which cyclizes to the corresponding ring structure. The enzyme is a flavoprotein as indicated by peroxide production. In the absence of catalase the accumulated peroxide further oxidizes the α-keto acids to the next lower carboxylic acid.

Hundredfold purification of a D-amino acid oxidase from a species of *Aspergillus* (18) has been achieved. In contrast to renal D-amino acid oxidase, the new enzyme is highly active for glutamic and aspartic acids, yielding the corresponding α-keto acids and NH_3 in the presence of catalase. In the absence of catalase, peroxide further oxidizes α-ketoglutarate to succinate and CO_2. The enzyme is sensitive to cyanide and PCMB but not to metal-binding reagents. D-amino acid oxidase purified from kidney and in crude brain homogenates was found to be 50 per cent inhibited by chlorpromazine ($2\times10^{-5}M$), which may prove to be a useful inhibitor (19). Greenstein and his colleagues (20) studied the susceptibility of the four diastereoisomers of threonine and isoleucine as substrates for several samples of ophio-L-amino acid oxidase and of two different D-amino acid oxidases. In general, the α-D, β-D configuration was most suitable as substrate for the D-amino acid and the α-L, β-L configuration for the L-amino acid oxidases.

The reactions in which ammonia may be fixed appear to be relatively restricted; the major paths involve the operation of glutamic dehydrogenase, glutamine synthetase, and the formation of carbamyl phosphate. Whereas K_m for ammonia in these three systems may be relatively high, the abundance of glutamate, ketoglutarate, CO_2, and ATP, together with the equilibrium positions of these reactions, are such as to assure relatively low cellular concentrations of NH_3-NH_4^+. Thus for glutamic dehydrogenase at pH 7, assuming a DPN-DPNH ratio of 1, glutamate and ketoglutarate concentrations of 3×10^{-3} and $1\times10^{-3}M$, respectively, and $K=2\times10^{-15}$ (21), the NH_4^+ concentration would be of the order of $2\times10^{-5}M$. Reliable data on the steady state concentration of ammonia in mammalian cells do not appear to be available for comparison with this calculated value.

Hoberman (22), in discussing the factors involved in the nitrogen-sparing action of glucose, considers that the major mechanism involves the availability of reducing power to increase the DPNH concentration and, by favoring NH_3 fixation into glutamate, renders NH_3 unavailable for urea synthesis. In the absence of data concerning the DPNH-DPN^+ ratio in fasted and glucose-fed livers, one might equally argue that the fasted liver drains protein for its glutamate, aspartate, and alanine into the tricarboxylic acid cycle under circumstances where there is no glycolytic production of pyruvate for

replenishment of the cycle by the malic enzyme or phosphoenolpyruvate carboxylase.

As might be expected from the calculation above, mammalian extracellular ammonia concentration is normally trivial. However, when the systems for fixing ammonia are overloaded, cellular and extracellular ammonia may increase markedly. Greenstein and his colleagues (23 to 28) have established the toxicity ($LD_{99.9}$) of many naturally occurring amino acids and their optical enantiomorphs. In order of decreasing toxicity they are tryptophan, arginine, lysine, histidine, threonine, methionine, phenylalanine, leucine, valine, isoleucine, glycine. In all cases, at toxic levels of administration there was a marked rise in blood ammonia concentration; the authors consider that death resulting from excessive amino acid administration reflects ammonia poisoning. This was supported by the observation that addition of L-arginine to infusions of the other amino acids or of NH_3 increased urea production, markedly reduced toxicity, and prevented a rise in plasma NH_3, thereby affording a demonstration *in vivo* of the operation of the Krebs-Henseleit cycle for urea production. Parenthetically it may be remarked that the toxicity of the basic amino acids appears to be excessive, considering other known aspects of the metabolism of these amino acids, and may in part reflect the fact that these amino acids were administered as their hydrochlorides. No explanation was offered for the interesting observation that administration of massive quantities of amino acids invariably led to a marked hyperglycemia which occasionally declined to hypoglycemic levels just before death. Similar findings in dogs given large amounts of glycine had been previously reported by Handler, Kamin & Harris (29). Noteworthy also is the fact that protection against toxicity of other amino acids was afforded by the administration of D-arginine, L-ornithine, L-citrulline, and α-acetyl-L-ornithine, but not by α-acetyl-L-arginine, δ-acetyl ornithine, glutamate, or glutamine. Those substances which protected against the toxicity of other amino acids also prevented the increase in ammonia and increased urea production. Without adequate explanation were the observations by the same authors (30) that in the presence of a subprotective level of L-arginine, a series of materials synergistically protected against ammonia toxicity. These included sodium glutamate, glutamine, sodium α-ketoglutarate, sodium aspartate, asparagine, sodium oxalate, alanine, sodium pyruvate, glucose, and sodium chloride. Particularly puzzling is the observation that ornithine could not substitute for arginine under these circumstances. Similar increments in plasma ammonia concentration after administration of glycine and, to a lesser extent, of alanine or casein hydrolysate to dogs were observed by Harper and his colleagues (31, 32) who also demonstrated that arginine is protective whereas sodium glutamate is not. The origin of plasma NH_3 in animals given massive doses of amino acids has not been established. It would be of interest to learn whether, under these conditions, the liver makes ammonia more rapidly than urea or whether the plasma NH_3 is derived from oxidations in the kidney or elsewhere.

Clinical interest in ammonia metabolism centers about hepatic disease and numerous investigators have noted large increments in plasma ammonia in cirrhosis, hepatitis, and liver damage secondary to biliary tract disorders (33, 34, 35) with increases in blood ammonia observed after protein feeding (36). An attractive hypothesis with respect to the mechanism of "ammoniagenic coma" has been offered by Bessman (37, 38, 39) who has observed that, with an elevated concentration of ammonia in the arterial plasma, ammonia is removed from blood flowing through the brain while venous glutamine appears in approximately equivalent amounts; brain glutamic acid decreases concomitantly. Since the only "NH_3-disposal system" available to brain is glutamine formation, and since glutamine can penetrate the blood-brain barrier in both directions, an elevated input of ammonia results in removal of α-ketoglutarate from the cellular pool available for the operation of the citric acid cycle. In contrast to liver and cardiac muscle, brain appears to be limited in its capacity to generate dicarboxylic acids from pyruvate; therefore the respiration of brain must fail under these circumstances, in keeping with the diminished oxygen consumption of brain tissue in ammoniagenic coma. Arginine was observed to diminish plasma ammonia in patients with hepatic disease (40) and to protect against the development of coma. These various findings and, indeed, the initial observations of Krebs & Henseleit suggest that the limiting factor in the manufacture of urea by mammalian liver is the steady state supply of ornithine and citrulline. Studies of arteriovenous differences for NH_3 across the liver and kidney (41) suggest that urea synthesis by cirrhotic liver is adequate, but only at elevated NH_3 concentration. The reported diminution in plasma ammonia in cirrhotic patients (33, 35, 40) after glutamic acid administration may reflect synthesis of glutamine which is stored temporarily as an alternative pathway of ammonia disposal.

Weil-Malherbe & Green (42) in extending earlier studies have observed that brain slices increase in glutamine content without change in ammonia concentration when incubated in the presence of glucose; in the absence of glucose, ammonia appeared. The latter process was inhibited by both anoxia and DNP. The authors conclude that these observations cannot be explained by the operation of known deaminating systems of brain. A partial explanation is afforded by the interesting properties of the brain adenylic deaminase system associated with insoluble particles; the system was purified twentyfold (43). The enzyme preparation was free of adenosinetriphosphatase, 5′-nucleotidase, myokinase, and adenosine deaminase; deamination of AMP was observed only in the presence of ATP; ADP, ITP, and IDP were inactive. The ATP was recovered at the end of the incubation period, confirming previous observations of Muntz (44). Since ammonia appearance in slices lacking glucose was not accompanied by diminution of glutamine concentration, NH_3 may, in part, be derived from adenylate by the operation of this enzyme which, like NH_3 production, was stated to be DNP-

sensitive. The possibility that this apparent DNP-sensitivity reflects a decrease in ATP concentration resulting from the usual uncoupling action of DNP should be investigated.

Renal ammonia secretion.—Richterich-van Baerle, Goldstein & Dearborn (45) reported that in the guinea pig ammonia excretion increases, not only in acute acidosis but in acute alkalosis. If a single mechanism is involved, this finding destroys the concept that ammonia secretion is merely an obligatory consequence of the acidification of urine with protons trapping the diffusible NH_3 which remains in equilibrium with the low steady state concentration of NH_3 in tubular cells. The possibility that an entirely separate device is operative in the alkalotic guinea pig has not been excluded but seems unlikely in view of this group's finding (46) that the glutaminase I (phosphate-activated) and glutaminase II (pyruvate-activated) activity of guinea pig kidney homogenates increase 100 and 40 per cent respectively in acidosis, and 250 and 70 per cent in alkalosis. These were paralleled by 60 and 90 per cent increases in glutamine synthetase activity in these two states, respectively. A similar "adaptive" increase in glutaminase I activity of kidney from acidotic dogs was recently reported by Muntwyler *et al.* (47) and confirms the earlier findings of Davies & Yudkin (48) with kidney slices of acidotic rats. Alkali administration slightly lowered the apparent renal glutaminase activity of dogs and rats (47, 48), in conformity with the fact that these species do not appreciably excrete ammonia in alkaline urines. It would be of interest to learn the "adaptive" behavior of renal glutaminase in the rabbit whose ammonia excretion is only slightly enhanced by acidosis. It should be noted also that in all these studies enzyme activity is determined: there has been no demonstration that the quantity of enzyme increases.

In another significant contribution (49), this same laboratory has compared the activity of the two glutaminases and of glutamine synthetase in the cortex, and outer and inner medulla of dog and rabbit kidneys. All three enzymes were most active in the cortex. However, although the cortex was 3.5 times as active with respect to glutaminase I as was the inner medulla, the total activity of the latter (largely collecting ducts) was sufficient to account for the maximum urinary excretion of ammonia in each species. It is not known whether the medullary glutaminase participates in the "adaptive" response to acidosis, but if the collecting ducts are uniquely the source of urinary ammonia, then the bulk of the renal glutaminase activity of the acidotic animal is unrelated to the ammonia secretory device. It may be significant that the collecting tubules were quite weak in glutamine synthetase activity and even in the cortex, these authors found that the activity of this enzyme was only 2 per cent of glutaminase I and 25 per cent of glutaminase II activities. This marked discrepancy, and the unexplained phosphate activation of glutaminase I, which has never been purified, suggest that the metabolic significance of this enzyme may be related to some process other than simple ammonia formation.

GLUTAMINE

The widespread and abundant distribution of glutamine and of an enzyme for its synthesis have frequently led to discussion of the metabolic role of this compound. Clearly, in higher plants glutamine may serve as a reservoir of nitrogen when the supply exceeds requirements for protein and other compounds (50). To a lesser extent this may be true of microorganisms and of animals. The phenomenon of γ-glutamyl transpeptidation suggested that the energy of the glutamine amide bond may be used for protein synthesis by transpeptidation or other means. However, this concept has largely been dispelled (51). The studies of Steward *et al.* (50), indicate that in carrot tissue, grown in culture, most of the intracellular glutamine resides in a pool which is not miscible with the material being used for active protein synthesis since, 24 hr. after adding glutamine-C^{14} to the medium, the specific activities of free glutamate and of protein glutamate were considerably larger than that of the total glutamine. Moreover, the specific activities of protein-bound glutamic and aspartic acid were found to be twice as great after administration of glucose-C^{14} as after an equal amount of activity as glutamine.

The role of glutamine as donor of nitrogen from its amide position into amino groups is of interest. Pogell (52) has found that the amino group of glucosamine-6-phosphate, formed in rat liver, is derived from the amide position of glutamine by a reaction presumably analogous to that found earlier by Leloir & Cardini in *Neurospora* (53). More recently Lowther & Rogers (55) have found that suspensions of *S. hemolyticus* T_{13} synthesize hyaluronate from glucose and glutamine; the latter could be replaced by ammonium glutamate but not when methionine sulfoxide was included in the medium. Cell-free extracts of this organism synthesized a glucosamine derivative from glutamine but not from ammonium glutamate. It may be assumed that an enzyme system analogous to that described by Leloir & Cardini is active in this organism.

Bentley & Abrams (56) have now demonstrated with N^{15} that the synthesis by extracts of rabbit bone marrow, of guanylic acid from xanthylic acid, ATP, Mg^{++}, and L-glutamine, which they had observed previously (57), involves direct transfer of the amide nitrogen of glutamine to the purine molecule. A similar reaction has been observed by Lagerkvist (58) with pigeon liver extracts; the mechanism of this reaction remains unknown. In contrast, Magasanik, Moyed & Karibian (59) have observed the formation of guanylic acid from xanthylic acid, in the presence of ATP and NH_3, by cell-free extracts of *A. aerogenes* and *E. coli*, although they have not definitely ruled out intervention of glutamine. In this system, ATP is converted to AMP plus PP_i, an intriguing reaction whose mechanism is not considered by the authors. Since Magasinik's studies (59, 60) indicate that guanine C-2 and its associated amino group are transferred to form the imidazole N-1 and C-2 of histidine, and Neidle & Waelsch (61) have indicated that N-1 of the histidine imidazole derives from glutamine, it may be that

glutamine is an intermediate in the formation of guanylic acid from inosinic acid, NH_3, and ATP (59).

According to Sloane *et al.* (62), glutamine is required by the complex inosinic acid transformylase reaction wherein inosinic acid plus glycine yield aminoimidazolecarboxamide ribotide plus serine. Pigeon liver extracts prepared by the method of Greenberg (63) were inactivated by treatment with norite. Reaction in a mixture containing IMP, glycine, leucovorin, and ATP only proceeded when glutamine was added. The glutamine requirement appeared to be considerable and indeed in excess of the amount of glutamine which might conceivably have been removed by the norite. Since glutamine could not be replaced by a mixture of glutamate, ammonia, and asparagine, the glutamine effect appeared quite specific, but its role in this reaction remains to be elucidated. In contrast, the formation of CTP from UTP by an enzyme from *E. coli* strain B requires ATP and NH_3. The latter could not be replaced by glutamine and in the presence of glutamine, $N^{15}H_3$ was used, undiluted, for CTP synthesis (502).

The amide nitrogen of glutamine is used as the source of two of the nitrogens of the purine ring (64, 65). N-9 arises in the over-all reaction: glycine + glutamine + PRPP → glycinamide ribotide (GAR) + glutamate (66 to 69). Since synthetic 5-phosphoribosylamine substituted for PRPP + glutamine (70) it is assumed that the over-all reaction occurs in two stages: (*a*) Glutamine + PRPP → phosphoribosylamine + glutamate; (*b*) glycine + ATP + phosphoribosylamine → GAR + ADP + P_i (69, 70, 71). In the absence of glycine and ATP, glutamine and PRPP reacted to liberate glutamate, PP_i and what is presumed to be 5-phosphoribosylamine (which degraded to ribose-5-phosphate and could not be isolated). Goldthwaite (70) has now speculated on the possible intermediate formation of glutamine ribotide which would be analogous to GAR and therefore of comparable stability. However, this compound was not found. The complete system was not observed to exchange PP_i^{32} with PRPP and so the mechanism of formation of 5-phosphoribosylamine remains imperfectly understood. The subsequent formation of GAR also appears to be without a known parallel. Since P_i is formed in the reaction, a glycyl adenylate, analogous to those which have been described for amino acid activation for peptide synthesis, seems unlikely in this case. However, some form of activated glycine is synthesized since, in the absence of other acceptors, when the enzyme is incubated with glycine, ATP, and hydroxylamine, the hydroxamic acid is formed. The formation of glycinamide ribotide appears to be reversible; when the enzyme is incubated with GAR + ADP + P_i, ATP and glycine appear (71). With PP_i^{32}, ATP^{32} is formed, but only in the presence of both GAR and ADP. When arsenate was included in the incubation mixtures, glycine appeared, GAR disappeared and ADP was unnecessary. The latter observations are compatible with the formation of glycylphosphate as the activated form of glycine for this reaction.

A fraction obtained from pigeon liver by alcohol and ammonium sulfate treatment has been found to yield formylglycinamidine ribotide upon incu-

bation with formylglycinamide ribotide, ATP, and glutamine (72). Nothing is known of the mechanism of this reaction but its formal resemblance to the formation of guanylic from xanthylic acid should be noted. Each of the three glutamine-requiring steps noted in purine metabolism, viz., formation of glycinamide ribotide (70), of formylglycinamide ribotide (72), and of guanylic acid (56) has been noted to be inhibited by azaserine. These observations suffice to account for many of the previous observations of abnormalities of purine metabolism in azaserine-inhibited *E. coli*, including accumulation of formylglycinamide ribotide and alleviation of azaserine inhibition by 4-amino-5-imidazolecarboxamide, adenine, and other purines, and by glutamine (73, 74).

The mechanism of the glutamine synthetase reaction has been studied with the aid of O^{18} in two laboratories (75, 76). In both cases it was found that O^{18} in the carboxyl of glutamate is transferred to orthophosphate derived from ATP and suffers no dilution from the oxygen of the medium. The simplest explanation offered by both groups is a nucleophilic displacement of the phosphorus of the terminal phosphate of ATP by glutamate on the enzyme surface, with intermediate formation of γ-glutamylphosphate. However, this compound has not been observed by previous students of this reaction. Synthetic γ-glutamylphosphate was prepared by Levintow & Meister (77), but could not be used by the purified enzyme in place of glutamate plus ATP. Whereas this result clearly eliminates participation of free γ-glutamylphosphate, it does not exclude the participation of an enzyme-bound form of this material. It may be noted that because of the great instability of γ-glutamylphosphate, these authors prepared its N-acetyl derivative and added this together with kidney deacylase to the reaction mixture to generate the desired γ-glutamylphosphate. There remains the possibility, considered by Kowalsky *et al.* (76), of a nucleophilic attack on phosphorylated enzyme rather than on ATP. The data are compatible with a series of reactions in which enzyme plus ATP form phosphorylated enzyme, with phosphate then being displaced by glutamate to give glutamyl enzyme and P_i, and the glutamyl enzyme attacked by ammonia to give glutamine and enzyme. In such a series of reactions, the last would leave O^{18} on a hydroxyl group of the enzyme, if such were the nature of the reaction; in the next cycle, phosphate displaced from the enzyme would become labeled with O^{18} left previously by the glutamate. There are several complicating contraindications to this hypothesis which was offered only as a model to rationalize the results with O^{18} which might be produced by any of various displacement reactions on the enzyme surface, providing that the active group on the enzyme contains a displaceable oxygen. Whatever the mechanism, a simple "glutamyl enzyme" does not appear to be involved. This emerges clearly from the fact that highly purified glutamine synthetase will only catalyze the "transferase" reaction, glutamine$+NH_2OH \rightarrow$γ-glutamylhydroxamate $+NH_3$, when ADP and orthophosphate are present in the reaction mixture

(78). In any case, the data clearly eliminate the intervention of a glutamyl ADP derivative. Yet Hansen & Hageman (79) have recently reported the isolation by column chromatography and paper electrophoresis of glutamyl and aspartyl derivatives of ADP from chicken liver. On hydrolysis they yield one mole each of AMP, P_i, and either aspartate or glutamate. Since both derivatives give a ninhydrin reaction the authors postulate the existence of β-aspartyl or γ-glutamyl bonds. The metabolic function of these compounds remains to be determined. Should the linkage prove to be α rather than ω in these derivatives, the possibility that they may be used for peptide synthesis remains.

An unusual system has been reported by Connell & Hanes (80), who have partially purified from pig liver an enzyme which catalyzes the conversion of γ-glutamylamides (such as γ-glutamylglutamate, -glycine, or -phenylalanine, as well as glutathione) to the corresponding amino acids and pyrollidone-carboxylic acid. This enzyme was separated from the "γ-glutamyltranspeptidase" of liver and the authors suggest that it be called "γ-glutamyl lactamase." Sachs & Waelsch (81) have described chemical syntheses designed to prepare the α- and γ-glutamyl thioesters of glutathione. Each procedure yielded a mixture of the α- and γ-derivatives and the authors suggest that attention be paid to the possibility of similar interconversion of α- and γ-glutamyl bonds in the course of metabolic processes.

In one of the first reports of studies done with a powerful new research tool (82), it was found that glutamine is an important nutrient for mouse fibroblasts and human Hela cells in tissue culture. Glutamate at high concentration, plus a mixture of the "unessential" amino acids, replaced a large fraction of the glutamine requirement.

Urea Synthesis

Evidence continues to accumulate that the Krebs-Henseleit cycle is the major if not the exclusive mechanism for urea synthesis in biological systems. Thus, free ornithine and arginine were observed in the tissues of representative members of Lepidoptera, Hymenoptera, and Orthoptera in all stages of development. Citrulline was evident in most but not all stages (83). Incubation of the whole tissue of each species *in vitro* showed arginase activity, thus suggesting the usual operation of this pathway. Rather surprisingly, the authors state that "without doubt the urea is utilized for formation of uric acid."

Nitrogen for urea synthesis is delivered either as carbamyl phosphate or aspartate. However, the enzymatic generation of carbamyl phosphate from ammonia is but imperfectly understood. The role of acyl glutamates remains to be clarified: Hall & Cohen (84) have isolated a factor from rat liver mitochondrial acetone powder which stimulates the synthesis of carbamyl phosphate in a system free of glutamate derivatives. Separation of ornithine transcarbamylase from the system responsible for carbamylation of aspar-

tate has been achieved by Lowenstein & Cohen (85). The latter enzyme was found to be widely distributed among animal tissue whereas the citrulline-synthesizing enzyme was more restricted. Acetylglutamate was required for the over-all reaction: $CO_2+NH_3+ATP+aspartate \rightarrow carbamyl\ aspartate$. It was not required for the single step: carbamyl phosphate+aspartate $\rightarrow$carbamyl aspartate+P_i. Crokaert, Jones & Lipmann (86) observed formation of two radioactive spots, determined chromatographically, when pigeon liver extract was incubated with C^{14}-carbamyl phosphate and either glutamine or aspartate, but not with a series of other amino acids; the nature of the products was not determined. Reichard & Hanshoff (87) have also separated aspartate carbamylase from the ornithine system and achieved approximately hundredfold purification of the aspartate carbamylating enzyme from *E. coli*. These authors have rigorously identified carbamyl phosphate with what had earlier been designated Compound X and obtained good stoichiometry for the reaction: carbamyl phosphate+aspartate $\rightarrow$carbamyl aspartate+P_i. K_m for aspartate (0.065 M) is surprisingly high but the equilibrium position is far to the right, as written; the evidence of kinetic reversibility, using inorganic P^{32}, was equivocal. In agreement with the findings of Lowenstein & Cohen the enzyme was found to be sensitive to *p*-chloromercuribenzoate and protected by glutathione. Failure of exchange of C^{14}-aspartate with carbamyl aspartate was taken as an indication that a carbamyl-enzyme is not an intermediate. The authors conclude that the transferase reaction involves a single displacement mechanism in which carbamyl phosphate and aspartate are first attached to the enzyme and then, by a nucleophilic attack of the N-atom of aspartate on the carbon atom of carbamyl phosphate, the C—O bond is broken simultaneously with the formation of the new C—N bond.

Detailed studies of a series of reactions analogous to those reported in the definitive studies of Ratner (see 88) on argininosuccinate, have now been published. Lieberman (89) has demonstrated a requirement for GTP for the reaction between IMP and aspartate with formation of adenylosuccinate. The reaction is specific for GTP, while L-aspartic cannot be replaced by the D-isomer, L-asparagine, L-glutamate, or L-glutamine. These studies confirm and extend the previous observations of Abrams & Bentley (56, 57). Carter & Cohen have achieved hundredfold purification of yeast adenylosuccinase which converts adenylosuccinate to AMP and fumarate (90). As a net process, therefore, the nitrogen of aspartate is used to form AMP from IMP, thereby completing the synthesis of AMP from its simplest precursors. An analogous reaction is the formation of an intermediate, presumed to be 5-amino-4-imidazole (N-succinylo) carboxamide ribotide, from aminoimidazole ribotide, aspartate, CO_2, and ATP. A second enzyme degrades the intermediate to aminoimidazole carboxamide ribotide and fumarate (91) completing the analogy with the formation and degradation of argininosuccinate.

Greenberg *et al.* (92, 93) have continued their studies of liver arginase

and have obtained a preparation which is homogeneous both by electrophoresis and sedimentation, with a molecular weight of 138,000. The enzyme exhibits a turnover number of 1.32×10^5 moles of arginine per mole of enzyme per min. at 25°. The gross amino acid composition, determined by column chromatography, is not noteworthy. Manganese, nickel, and cobalt ions stabilized heat-treated arginase whereas zinc proved to be a strong inhibitor. The pH optimum was stated to be 10.2 with the acid branch of the pH curve determined by two acid-dissociating groups with pK's of 7.9 and 8.75. At the optimal pH for the enzyme, the substrate is a *zwitterion*. Carpenter & Pierce (94) have reported that a preparation of beef liver arginase of unstated purity yielded urea from a series of arginyl peptides, but only from those peptides which could also be hydrolyzed by the preparation (which contained peptidase); thus, the enzyme remains specific for free arginine. Mourgue & Baret (95) examined a series of guanido acids in the hope of finding a nonmetabolizable arginase inhibitor, but without success. Among the compounds tested were the guanido derivatives of butyric, propionic, and valeric acids as well as the corresponding α-hydroxy acids. The useful inhibitors available to date remain lysine, ornithine, homoarginine, and hexahomoserine.

Bronk & Fisher (96, 97, 98) have re-examined the fact, noted originally by Krebs & Henseleit, that ornithine appears to be a better catalyst for urea synthesis by liver slices than does citrulline. Since the present study indicated greater permeability of liver cells to citrulline than to ornithine, these authors considered the possibility that ornithine and citrulline catalyze separate processes both of which lead to urea formation. As a result of a series of complex experiments in which the relative amounts of ammonia, ornithine, and citrulline were varied and the rates of urea production as well as the tissue concentrations of ornithine and citrulline examined, the authors suggest two separate cyclic pathways whose steps are not specified. However, the complexity of the experiments and the differences noted scarcely seem to warrant serious consideration of an alternate to the Krebs-Henseleit pathway. The apparent superiority of ornithine as a catalyst for urea synthesis in such experiments remains without adequate explanation. Similarly, the observation that arginine and citrulline support the growth of *Drosophila* whereas ornithine is inhibitory (99) may not be regarded as compelling evidence against the existence of an ornithine-citrulline-arginine relationship but may, in part, reflect the inhibition of arginase by ornithine.

The burden of proof that there may exist a distinct pathway for urea synthesis via glutamine also remains with those who espouse this cause. This possibility arose with the experiments of Leuthardt (100) which formally resembled those of Krebs & Henseleit, viz., addition of glutamine to liver slices resulted in considerable enhancement of urea production from ammonia. Although this concept has never received support by isolation of appropriate enzymes or potential intermediates, it has frequently reappeared

in the literature. It was reconsidered by the present reviewers some years ago when it was observed that administration of glutamine to dogs producing urea maximally from infused arginine doubled the rate of urea production (101). A separate glutamine path has again been proposed by Bach & Smith (102, 103), who found that the maximum rate of urea synthesis by rat liver slices from glutamine was twice that from ammonia. However, at low concentrations, ammonia was decidedly superior to glutamine; at high concentrations of extracellular ammonia, the latter appeared to be inhibitory. Bromcresol green and bromcresol purple, at concentrations which elicited 60 to 80 per cent inhibition of glutaminase activity in liver homogenates, were without effect on urea synthesis by liver slices but it was not demonstrated that the dyes penetrate hepatic cells sufficiently to inhibit intracellular glutaminase. Pyruvate, added to potentiate glutamine hydrolysis by the transaminating system, actually abolished urea formation. Although this finding is rather surprising, sufficient pyruvate was added to account for a considerable fraction of the nitrogen involved by alanine accumulation. Without apparent explanation is the observation that urea formation from glutamine was entirely dependent upon CO_2; when the slices were suspended in Tris or phosphate buffer urea synthesis did not occur and the added glutamine was quantitatively recovered. Comparable data were not presented for urea synthesis from NH_3. Failure of glutaminase activity under these conditions remains unexplained. However, the totality of these observations, while leaving much to be explained, does not appear convincing as an argument in favor of a separate route for urea synthesis via glutamine. The special virtues of glutamine appear to be its ready permeability to liver cells, thus providing ammonia on hydrolysis, a source of glutamate for acetylglutamate formation and for transamination to maintain a high intracellular aspartate concentration, and perhaps a ready source for synthesis of additional ornithine. It will be recalled that the details of the path, glutamate →ornithine, are not at present known. These comments apply also to the observation that whereas there is no essential difference in urea production from glutamine by normal and hepatic tumor-bearing rats, the latter produced much less urea from glutamic acid than did normal animals (104).

Ellner & Steers (105) have confirmed the earlier observation of Davis (106) that *Chlorella* can use urea in place of nitrate as a nitrogen source but that urea carbon does not suffice as a carbon source. However, the carbon of urea-C^{14} was utilized in the presence of the added CO_2 necessary for growth. Indeed, under these conditions, about 50 per cent of urea carbon was fixed by growing *Chlorella* and *Schenedesmus*. The authors did not discuss their finding that guanine, formed under these circumstances, exhibited a specific activity 200 times that of the CO_2 present, strongly suggesting that the initial reaction in which the urea is degraded is something other than the classical urease-catalyzed hydrolysis.

Amidine compounds.—It is now abundantly clear that kidney contains

a transamidinase which has been partially purified by Fuld (107), Walker (108), and Ratner & Rochovansky (110). The enzyme has been demonstrated to catalyze transfer of the amidine group from arginine, guanidoacetic acid, and canavanine to glycine, ornithine, and canaline. Each possible combination of donor and acceptor has been observed to interact at appreciable rates including arginine and ornithine-C^{14} (yielding arginine-C^{14}), and arginine and lysine (to yield homoarginine). In each case the equilibrium constant is not far from 1 so that the "energy level" of the C—N bond involved in the reaction is of the same order for each of these amidine compounds although there is appreciable difference in reaction rates. Both Fuld and Walker have suggested the intermediation of an enzyme-amidine complex as the immediate amidine donor, although the nature of the link between amidine and enzyme was not specified. However, studies of isotope incorporation and of the kinetics of product inhibition led Ratner & Rochovansky (111) to suggest that transamidination occurs by a displacement reaction with donor and acceptor attached to the enzyme surface at two adjoining but equivalent binding sites in the catalytic area. This seems particularly attractive since competition for the enzyme was demonstrated between pairs of acceptors, pairs of donors, and between acceptors and donors. These authors rejected the concept of an enzyme-amidine intermediate. Although the reaction proper is quite different, the mechanism suggested recalls the displacement mechanism suggested earlier for phosphate transferases by Boyer & Harrison (112), and by Reichard & Hanshoff (87) for aspartate transcarbamylase. Despite the formal resemblance between transcarbamylation and transamidination there has been no evidence of analogous formation of an amidine phosphate. Each of these investigators has observed weak arginase activity in their transamidinase preparations. If this proves to be associated with the purified enzyme, rather than being a contaminant, any proposed mechanism must account for this property which is more readily reconciled with an "amidine-enzyme" than with a simple displacement reaction between the two substrates.

Walker (113) has also observed the formation of canavaninosuccinate from canavanine and fumarate by kidney preparations under conditions analogous to the formation of argininosuccinate from fumarate and arginine, as described by Ratner. The formation of this new compound was observed with preparations from jackbeans, *Chlorella*, *E. coli*, and various lactobacilli. Since the formation of this compound was inhibited by arginine and that of argininosuccinate by canavanine the author concludes that argininosuccinase was responsible for both reactions. In establishing the structure of canavaninosuccinate, the latter was reduced with hydrogen to homoserine and guanidine. A similar reductive but enzymatic cleavage has been observed in the case of canavanine itself which is hydrogenolyzed to homoserine and guanidine by resting cells of *S. faecalis* (114). Since a pathway is available for the conversion of homoserine to threonine, canavanine can be used in

place of threonine in the nutrition of threonineless *Neurospora*. The mechanism of this enzymatic reduction remains to be established.

Two groups have studied transfer of the amidine group *in vivo*. Horner, Segal & Bruton (115) fed rats guanidoacetic acid, with C^{14} in the amidine group, and recovered the isotope in urea with a specific activity considerably greater than that of the CO_2 concomitantly respired, thus indicating transfer of the amidine group from guanidoacetic acid to arginine without isotope loss. Stetten & Bloom (116) fed arginine labeled with both C^{14} and N^{15} in the amidine group to rats for three days. The isolated creatine contained N^{15} and C^{14} in a ratio identical with that in the arginine fed, thus providing an elegant demonstration of the intact transfer of the amidine group *in vivo*. Incidentally, it was observed that the ratio N^{15}/C^{14} in the excreted urea was approximately equal to that in the administered arginine on the first day but considerably higher thereafter. The authors ascribe this to hydrolysis of urea in the gastrointestinal tract. Both the ammonia and carbon dioxide resulting therefrom can be reutilized for urea synthesis, but since the ammonia is diluted by a much smaller pool than is the carbon dioxide, the N^{15}/C^{14} in the final urea is considerably greater than in the original arginine.

Smith (117) has described an improved synthesis of amidine-labeled citrulline and used the procedure for synthesizing the analogous C_4 and C_6 compounds. Both proved inactive for the "citrullinase" of liver and were rather poor inhibitors when citrulline itself was the substrate. This author has confirmed the unusually strong product inhibition of citrullinase by ornithine, observed earlier by Krebs *et al.* (118).

Pyridoxal Phosphate, Transamination, and Decarboxylation

The year has witnessed increased use of isonicotinyl hydrazide for the detection of enzymes employing pyridoxal phosphate. Bacterial lysine and diaminopimelic acid decarboxylases (139), ω-aspartic decarboxylase of *Desulfovibrio desulfuricans* (140), hepatic glutamic-alanine transaminase, and the glutamine-pyruvate transaminase-deaminase have all been observed to be inhibited by this reagent whose use has been summarized by Meister & Downey (141) and Davison (142). Inhibition of glutamic-alanine transaminase was markedly increased by Cu^{++} (143), indicating formation of a chelate of the hydrazone of INH and pyridoxal phosphate, similar to the metal chelates suggested by Metzler, Ikawa & Snell (144) between amino acids and this coenzyme. Davison (142) has provided direct evidence for the formation of the hydrazone and studied the kinetics of INH inhibition.

Binkley & Boyd (145) have extended the previous observations of Metzler *et al.* (144), observing that a chelating agent markedly enhances the cleavage of cystathionine by pyridoxal phosphate and a metal. Further support for the mechanism of pyridoxal phosphate action proposed by Metzler *et al.* comes from the observation that pyridoxal, plus a divalent metal, nonenzymatically catalyzes dehydrochlorination of β-chloroalanine with the formation of ammonia, pyruvate, and chloride—presumably by β-elimination. A rat liver preparation, which required pyridoxal phosphate for maximal

activity, catalyzed the same reaction and also exhibited homoserine dehydrase activity (146).

A number of new transaminases have been demonstrated which transfer nitrogen from glutamate to imidazoleacetol phosphate, to dihydroxyfumarate (presumably as oxaloglycolate) and to glyoxylate with formation of histidinal phosphate (147), hydroxyaspartate (148), and glycine (149) respectively. Kynurenine transaminase has been partially purified both from rat kidney (150) and *Neurospora* (151) and demonstrated to require pyridoxal phosphate for maximal activity. Fincham & Boulter (152) have presented evidence for the existence in *Neurospora* of a minimum of four independent transaminases with varying specificities; one of these is relatively unspecific, accepting nitrogen from at least nine amino acids with formation of glutamate. Chicken liver has been stated (153) to contain another relatively unspecific transaminase which transfers nitrogen among any two of the following: leucine, isoleucine, valine, methionine, α-aminobutyrate, and glutamate, thus resembling an enzyme earlier reported in *E. coli*. The enzyme was sensitive to sulfhydryl reagents and was activated by sulfhydryl compounds but it was not possible to establish a requirement for pyridoxal phosphate. Rowsell (154) has suggested the presence in rat liver of one or more transaminases capable of transferring from various amino acids to pyruvate without the intervention of glutamate. He has demonstrated (155) that the rate of transamination with α-ketoglutarate by rat liver exceeds the formation of ammonia plus urea by rat liver slices, supporting the concept that the combination of transaminases and glutamic dehydrogenase suffices to account for oxidative deamination of amino acids by liver.

Canellakis & Cohen (156) have considerably purified the tyrosine-ketoglutarate transaminase of liver. The active protein is bright blue and gives the emission spectrum of copper but is not inhibited by versene or pyrophosphate. The kinetics and substrate specificity of the enzyme, which exhibited a K_m for pyridoxal phosphate of $10^{-7}M$, were studied in detail. A large series of compounds structurally related to tyrosine were examined as both substrates and inhibitors, permitting a rather exact description of the structural specificity requirements of this enzyme (157). Hilton *et al.* (158), continuing studies of the mechanism of aspartate-ketoglutarate transamination with the use of N^{15} and D_2O, have observed that the rate of entry of deuterium into aspartate exceeds the rate of transamination and that deuterium entry is depressed by increased concentrations of ketoglutarate. Using aspartate-N^{15} in D_2O, D/N^{15} found in the glutamate was 1, indicating participation of the α-hydrogen atom in the exchange. Tanenbaum (159) has found that N^{15} in the amino group of various amino acids is not diluted by NH_3-N in the medium during transamination to ketoglutarate. The preparation (from pig heart) also transferred N^{15} from pyridoxamine to glutamate. Interpretation of this finding is difficult although the author suggests that pyridoxamine may behave as a substrate for the enzyme which already contains bound pyridoxal phosphate.

In an important communication, Longenecker & Snell (160) report that

the L-isomer of glutamate is preferentially formed when ketoglutarate is transaminated nonenzymatically by Cu^{++} plus pyridoxal and either L-alanine or L-phenylalanine. Similarly, D-glutamic acid was preferentially formed from the D-somers of both amino acids. These observations provide direct evidence for the postulated participation of metal chelates as intermediates in these reactions and demonstrate that the chelates are asymmetric in structure. If metal ions participate in enzymatic transamination, formation of similar asymmetric chelates between enzyme and substrate may in part explain the optical specificity of the transamination reaction. Hurwitz (161) found that some pyridoxine analogues served as competitive inhibitors with pyridoxal for pyridoxal kinase while others, which served as substrates for pyridoxal kinase, yielded products which competed with tyrosine apodecarboxylase. Those analogues with a substituent at C-6 were not phosphorylated, whereas those unsubstituted at C-6 and containing a 5-hydroxymethyl group served as substrate for the kinase.

Glutamic acid decarboxylase of *Rhodotorula glutanis* has been partially purified (162). The fieldbean was noted to be extremely rich in this enzyme (163) and may be a good source for large scale preparation. A simple extract of *Mycobacterium* was noted to be rich in L-glutamic decarboxylase and devoid of decarboxylase activity for any one of 18 other amino acids; it did not contain glutaminase, thus providing a simple source of enzyme for glutamic acid determinations (164). Pyridoxal phosphate was established as coenzyme of 5-hydroxytryptophan decarboxylase (165) by the use of preparations from deoxypyridoxine-fed rats. A strain of *Desulfovibrio desulfuricans* was noted to convert aspartate to L-alanine (166); presumably, the responsible enzyme resembles that previously demonstrated in *Clostridium welchii* (167). Without explanation is the observation (168) that benzene in low concentration appears to activate histidine decarboxylase of rabbit kidney. A potentially useful tool was introduced by Michel & François (169) who noted that chlortetracycline inhibited decarboxylation of a series of amino acids by the mixed bacterial flora of pig intestine.

Of interest is the finding that both D-glutamic acid (170) and D-alanine (171) are constituents of the cell wall of lactic acid bacteria, thus providing a rational basis for the existence of racemases in these organisms. Finally, it may be noted that Davison (172) has observed that partially purified diamine oxidase is progressively inhibited by INH and reactivated by pyridoxal phosphate, suggesting that it may be the pyridoxal phosphate chelate of the diamine substrate which loses electrons to the presumed flavin of this enzyme.

Amino Acid Transport

Several papers have provided substantial support for the concept that amino acid entry into cells is an active, energy-requiring process. Thus, isolated intestinal loops actively take up L- but not D-phenylalanine. The same preparation takes up histidine from the medium; this process is inhibited by

anoxia and the amino acid is released by the addition of DNP (173). Likewise, it was found (174) that during experimental shock a condition resembling anoxia is established in the intestine and results in parallel failure of absorption of glucose, fructose, alanine, leucine, and glutamine. Sacs of everted small intestine of the hamster have been shown to transfer many amino acids actively against a concentration gradient (175). Of nine amino acids tested, only tryptophan did not appear to be actively transported. Similarly, slices of carrot were seen to take up amino acids from the medium and to establish a large concentration gradient in favor of the tissue; this process was inhibited by DNP and by cyanide (176). Both D- and L-isomers were removed from the medium although the rate of uptake of the L-isomers considerably exceeded that of their D-enantiomorphs. Although the energy requirement for this transport is indisputable, a considerable difficulty arises in interpretation of such data, since the studies of Cowie & Walton (177) suggest that a considerable portion of what is measured as the "free" amino acids of the cell may be loosely but specifically bound. Perhaps another indication of this phenomenon comes from the kinetic studies of glycine incorporation into *E. coli* by Koch (178) who has found that, at sufficiently high concentrations, glycine uptake became independent of concentration but, and probably more significant, these cells never take up all of the glycine-C^{14} from the medium. Unfortunately this residual glycine was not specifically identified as such and was noted only as radioactivity.

Competition among various transporting systems continues to be studied. Recently Mandelstam (179) has reported that putrescine and cadaverine inhibit the passage of ornithine and lysine, respectively, into *B. cadaveris*, as indicated by the rate of decarboxylation of the latter acids, although the diamines have no effect on the cell-free decarboxylases. Inhibition of this organism by the diamines was competitively overcome by the respective diamino acids. In *E. coli*, inhibition of growth by α-amino butyrate was overcome by alanine or leucine. Inhibition by β-amino butyrate was overcome by β-alanine while inhibition by α,γ-diamino butyrate was overcome by ornithine and, noncompetitively, also by methionine, methionine sulfoxide, homocystine, and arginine, but not by glutamate (180). These diverse effects may find their explanation in inhibition of a transport system rather than inhibition of intracellular enzymes.

Christensen has continued his studies of the possible role of pyridoxal derivatives in amino acid transfer and has continued to accumulate information compatible with this hypothesis. Thus, the structural features of an amino acid which influence biological transfer into the Ehrlich ascites cell also were found to modify reaction with pyridoxal in the presence of metals in a manner consistent with the participation of a metal chelating pyridoxylidene derivative of the amino acid in the transfer process. α-Substitution of the amino acid, which would prevent tautomerization of the pyridoxylidene derivative, intensified its transfer. Similarly, in comparing the effect of a group of aromatic *o*-hydroxy aldehydes in stimulating amino acid transfer,

it was found that those which form the most stable chelating Schiff's bases with amino acids were most effective in stimulating transfer (181). Chelates of several metals with the pyridoxylidene derivatives of α,γ-diamino butyric acid were prepared in the solid state and found to contain one metal atom for two Schiff base residues, thus yielding an unusually stable structure. This may be correlated with the fact that this compound is so strongly concentrated by ascitic cells as to be destructive to them (182). Three α-substituted amino acids which are metabolically inert (α-amino isobutyric acid, α-methylolserine, and α-methylserine) were shown to be accumulated by rat liver after intraperitoneal administration and were only excreted to a trivial extent in subsequent days (183). All three yield stable pyridoxal derivatives which form stable metal chelates, but, lacking α-hydrogen, cannot tautomerize and presumably therefore cannot be further metabolized. Christensen has also suggested that the amino acid pyridoxal metal chelates may also function in transport of metal ions (184), specifically with respect to the maintenance of a high intracellular potassium concentration in the presence of extracellular sodium. Under various circumstances intracellular potassium exchanges for a basic amino acid. Thus, erythrocyte cells which have concentrated α,γ-diaminobutyrate do so in exchange for potassium which is ejected (185). Similarly, lysine and arginine increase in the tissues of potassium-deficient rats (186) and it has been suggested that lysine may enter *B. cadaveris* freely but is ejected in exchange for potassium by the transport device (179). Perhaps related to these problems is the observation by Castel (187) that *N. crassa*, grown on Sr^{++} instead of Ca^{++}, releases glutamate, threonine, alanine, and valine into the medium. The release of proline into the medium of *Sarcina lutea* in the presence of penicillin (188) is considered by Brown & Binkley to be analogous with the failure of glutamate transport induced by penicillin. A preliminary report by Frieden (189) indicates that mouse kidney slices take up glycine-C^{14} from the medium at low concentration much more efficiently if the animal is pretreated with testosterone. At high glycine concentration this difference is not apparent. The situation is strikingly similar to the effect of insulin on glucose uptake by diaphragm.

METABOLISM OF AMINO ACIDS

Glycine-Serine Transformations

"*Serine aldolase.*"—It is now firmly established that serine is a glycine precursor, and that the two amino acids are interconvertible through transfer of a C_1 unit. Numerous isotopic and nutritional studies have established that glycine can be derived from the carboxyl and α-carbon of serine, and that the β-carbon of serine can both be derived from a C_1 unit, and can yield a C_1 unit which can be used to synthesize purines, methyl groups, etc. The observation that folic acid derivatives can exist in hydroxymethyl and formyl forms has suggested that this vitamin acts as cofactor in C_1 transformations; this has been borne out by numerous studies in the whole animal and in isolated enzyme systems.

Thus, the pathways have been established and the precise enzymatic mechanisms which mediate these transformations currently engage the attention of workers in this field. The enzyme complex which catalyzes the reaction serine $\rightleftharpoons$glycine$+C_1$, termed "serine aldolase" by Huennekens *et al.* (227) and Alexander & Greenberg (228), has been partially resolved; and the mechanism whereby THFA acts as a C_1 carrier has become considerably clarified. Separate enzymes appear to be required for initial formation of a serine-THFA complex, for splitting of this complex to glycine and hydroxymethyl-THFA (pyridoxal phosphate-requiring step), for removal of the C_1 unit from THFA, and probably for interconversion of various folic acid derivatives.

A requirement for pyridoxal phosphate, previously suggested (229, 230), has now been demonstrated. Alexander & Greenberg (228) have observed an absolute requirement for pyridoxal phosphate for serine synthesis by a partially purified enzyme from sheep liver; this requirement was not evident for serine decomposition by the same preparation. The reaction required THFA, and the authors calculated $\Delta F = -4900$ cal. for the condensation. The necessity of pyridoxal phosphate for serine synthesis from glycine has also been demonstrated with preparations from beef liver (227), rabbit liver (231), acetone dried luminous bacteria (232), and *Clostridium HF* (233, 234). Although two studies (228, 231) with mammalian liver failed to demonstrate a metal requirement, the more highly resolved pigeon liver system of Jaenicke (235) required manganese as well as pyridoxal phosphate.

The role of pyridoxal phosphate in the conversion of serine to glycine has been elucidated by Jaenicke (235) who fractionated a pigeon liver enzyme system, which had previously been demonstrated (236) to transfer the β-carbon of serine to THFA with subsequent oxidation to N^{10}-formyl-THFA. Fraction-1, in presence of pyridoxal phosphate, Mn^{++}, and THFA, formed hydroxymethyl THFA and glycine. After further fractionation of fraction-1 and treatment with charcoal to remove pyridoxal phosphate, a folic acid derivative, "X," which gave a ninhydrin color, was formed from serine and THFA. Acid hydrolysis or treatment with unfractionated fraction-1 converted "X" to glycine and a compound which gave a formaldehyde reaction. When unfractionated enzyme (1 plus 2), pyridoxal phosphate, and TPN were present, N^{10}-formyl-THFA and glycine were formed from compound "X." Fraction-2, in the presence of enzymatically or nonenzymatically formed hydroxymethyl THFA and TPM, produced N^{10}-formyl-THFA. The over-all reaction may be formulated as:

1. (*a*) serine + THFA $\rightleftharpoons$ THFA — serine

   (*b*) THFA — serine $\xrightleftharpoons{\text{pyridoxal phosphate, } Mn^{++}}$ hydroxymethyl-THFA + glycine

2. hydroxymethyl — THFA + TPN $\rightleftharpoons$ N^{10}-formyl-THFA + TPNH + H^+

Thus, pyridoxal phosphate appears to be required for the scission of a serine-THFA complex to glycine and hydroxymethyl — THFA. It is of considerable interest that THFA — serine, if its existence is confirmed, is the first product

observed wherein a folic acid derivative is condensed with a compound of more than one carbon.

Although the final product of the above reaction sequence is N^{10}-formyl-THFA, it is not known whether initial condensation of serine occurs in the N^5 or N^{10} position of the pteridine nucleus, nor is the location of the hydroxymethyl group clearly established. Kisliuk (237) has demonstrated that THFA binds formaldehyde-C^{14} nonenzymatically to form a product which gives two spots on a chromatogram; the bound formaldehyde can be converted to serine in the presence of a pigeon liver preparation and glycine, without mixing with a free formaldehyde pool. Doctor & Awapara (238) observed incorporation of serine β-carbon into citrovorum factor by extracts of chicken liver acetone powder, and suggested that hydroxymethyl-THFA and N^{10}-formyl-THFA were intermediates (cf. 236, 239).

Although most studies indicate that THFA is the C_1 carrier between serine and glycine, a different folic acid derivative may assume this role in *Clostridium HF* (233, 234). THFA and citrovorum factor mediate the serine to glycine transformation in this organism, but a polyglutamic folic acid derivative ("Co-C") from extracts of *Cl. cylindrosporum* is active in much smaller quantities. It should be noted that the incorporation of glycine-C^{14} into serine in extracts of luminous bacteria (232) required substrate, rather than catalytic, quantities of THFA. The role of various folic acid derivatives in C_1 transfer has recently been reviewed (240).

The final step in the "serine aldolase" reaction represents the scission of a C_1 fragment from THFA; scission of N^{10}-formyl-THFA appears to involve esterification of P_i and formation of ATP (6, 7). The reverse reaction, formation of N^{10}-formyl-THFA from formate, had been shown to require ATP (236, 239, 241). The requirement for P_i and ADP for formation of formate from N^{10}-formyl-THFA will be discussed under "formimino group transfer," where this reaction was observed.

Another type of reaction which can interconvert glycine and serine is a transformylation involving IMP, observed in chicken and pigeon liver by Flaks & Buchanan (242) and Warren & Flaks (243). This reaction, for which a folic acid derivative is required, is: IMP+glycine+TPNH+$H^+$$\rightleftharpoons$AICAR +serine+TPN; a similar reaction utilizing glycinamide ribotide and IMP to form FGAR has also been observed. These reactions could well contain steps in common with the "serine aldolase" reaction.

Formimino group transfer.—Formimino compounds have previously been observed as intermediates in histidine degradation [formiminoglutamic acid (244)] and in purine degradation [formimino glycine (FIG) (245)]. These studies have now been extended; it appears that a —HC=NH group can be transferred intact in a manner analogous to —HC=O, via a new folic acid derivative, formimino-THFA.

These findings have emerged from studies on FIG degradation to glycine, NH_3, and formate in *Clostridia* (5 to 11), and from studies on formimino

glutamate degradation by mammalian liver extracts (2, 3). Both laboratories studying FIG degradation in *Clostridia* agree that THFA is required and that the initial step is the transfer of a formimino group from FIG to THFA to yield formimino-THFA (8, 11) and glycine. Rabinowitz & Pricer (8) suggest initial formation of 5-formimino-THFA, with subsequent removal of NH_3 in the process of forming a 5,10-methenyl-THFA, which subsequently yields N^{10}-formyl-THFA. Phosphorylation of ADP in the process of degradation of FIG to glycine, formate, and ammonia has been observed (6, 7); this phosphorylation appears to accompany the degradation of N^{10}-formyl-THFA to formate and THFA. Thus, the over-all reaction (neglecting THFA rearrangements) may be written as:

$$(a)\ \text{FIG} + \text{THFA} \rightleftharpoons \text{NH=CH—THFA} + \text{glycine}$$
$$(b)\ \text{NH=CH—THFA} \rightarrow N^{10}\text{-formyl-THFA} + NH_3$$
$$(c)\ N^{10}\text{-formyl-THFA} + \text{ADP} + P_i \rightleftharpoons \text{THFA} + \text{formate} + \text{ATP}$$

$$\text{FIG} + \text{ADP} + P_i \rightarrow \text{glycine} + \text{formate} + NH_3 - \text{ATP}$$

The phosphorylation which accompanies breakdown of N^{10}-formyl-THFA is reversible (7); indeed the equilibrium position is far to the left. This finding is probably pertinent to the observations (236, 239, 241) that ATP is required for the incorporation of radioactive formate into serine and amino-imidazole carboxamide ribotide via N^{10}-formyl-THFA.

Sagers *et al.* (11) have suggested a similar degradative mechanism for formimino glutamic acid formed in histidine degradation. That this is indeed the case is evident from studies of Miller & Waelsch (2) and Tabor & Rabinowitz (3); both groups of workers have reported formation of N^{10}-formyl-THFA from formimino glutamate by mammalian liver extracts; the mechanism appears to be similar to FIG degradation. If formimino-THFA is indeed an intermediate in formimino group transfer, this conjugate may provide a means for the transfer, as a unit, of the carbon and nitrogen of the C_2 and amino group of guanine to the imidazole ring of histidine, as observed by Magasanik (59, 60). Whether guanine may be synthesized by ring closure with insertion of a C—N unit into aminoimidazole carboxamide ribotide, in a manner analogous to ring closure with "C_1" in inosinic acid synthesis, remains to be seen.

Several observations in the field of formimino group metabolism still require explanation: the report of Knudson (246) that folic acid inhibits glutamic acid formation from formimino glutamate by rat liver; the observation of Rabinowitz & Pricer (9) that the oxidation of glycine to CO_2 and acetate by *Cl. acidiurici* is dependent upon the presence of catalytic quantities of FIG; and that of Wachsman & Barker on the formation of formamide in histidine fermentation by *Cl. tetanomorphum* (247).

Biosynthesis of glycine and serine.—Studies of the neogenesis of glycine and serine focus upon the origin of the serine carbon chain (51, 248). The pathway proposed by Ichihara & Greenberg (249) (3-phosphoglycerate→

phosphohydroxypyruvate→phosphoserine→serine) has received additional support. Arnstein & Keglević (250) have compared alanine and glucose as precursors of serine and glycine in the guinea pig. The three-carbon serine precursor was found to be more closely related to triose or glyceric acid than to pyruvate; equal utilization of uniformly labeled and C_1-labeled glucose indicated that this precursor could not have been formed by the pentose pathway, where C_1 is lost as CO_2. Koeppe *et al.* (251) similarly concluded that a three-carbon glycolysis intermediate, other than pyruvate, was a serine precursor in the rat. However, Davis *et al.* (252), measuring the sequence of appearance of $C^{14}O_2$ into different amino acids of yeast protein, concluded that early appearance of radioactivity in serine and glycine (as compared to alanine) is consistent with their previous conclusions (253) that a glycine precursor is formed by α-β cleavage of a symmetrical four-carbon intermediate. The earlier studies reported conversion of pyruvate-2-C^{14} to glycine and serine, labeled equally in the carboxyl and α-carbon, but not in the β-carbon of serine. If glycine is a precursor of serine in yeast, the origin of the serine β-carbon is obscure; it could not arise from 1,2-labeled glycine, since this would lead to labeling of the β-carbon of serine.

Arnstein *et al.* (250, 254) have obtained additional *in vivo* corroboration of the known role of folic acid derivatives in mediating glycine-serine transformations, by demonstrating decreased serine to glycine conversion in folic acid-deficient guinea pigs; their studies also indicated that serine is the major source of glycine in this species. Similarly, Vohra *et al.* (255) observed that folic acid deficiency decreases the conversion of glycine to serine by livers of turkey poults.

The interconversion of glycine and serine must be of particular significance in the nitrogen economy of the bird. Birds suffer a daily uric acid-N loss equivalent to total nitrogen intake; 25 per cent of this nitrogen arises directly from glycine, and all carbons but one arise from glycine or serine or both (two directly from glycine, and two from formate, for which glycine and serine are probably predominant precursors). It is probably this fact which conditions the essentiality of glycine for the bird, and proportionality of glycine requirement to protein intake. Since dietary protein cannot supply adequate glycine plus serine, the bird must have, in addition to known mechanisms for transforming glycine to serine, major pathways of serine biosynthesis from other precursors. It is of interest that serine neogenesis has not been studied in birds, where this pathway must have greatest quantitative significance. A hen oviduct preparation which fixed $C^{14}O_2$ into the carboxyl group of acetate was also observed to fix CO_2 into glycine carboxyl (256); however, carboxyl-labeled acetate failed to yield carboxyl-labeled glycine; thus, the pathway of CO_2 into glycine remains obscure.

Further reactions of glycine, serine, and "C_1".—Sime, Dinning *et al.* (257, 258) observed that vitamin E deficiency in rabbits markedly increased the incorporation of formate into nucleic acid, methionine, and serine; formation

of CO_2 from formate was depressed, while $C^{14}O_2$ formation from glycine-1-C^{14} was stimulated. No change in glycine-serine interconversion was noted. Although the mechanism of these changes is completely obscure, a block in formate oxidation in vitamin E deficiency might provide partial explanation. Effects in some way similar were noted in surviving uterine strips of rats pretreated with estradiol (259); incorporation of serine-3-C^{14} into purines and proteins, and CO_2 production from serine, were markedly stimulated. Trapping of serine β-carbon in an unlabeled formate pool indicated that formation of formate from serine was accelerated. Whether the mechanisms of the effects of vitamin E deficiency and estradiol treatment are similar remains to be elucidated.

Stadtman & Elliott (12) have observed formation of ATP in the course of reductive deamination of glycine to acetate and NH_3 by preparations of *Cl. sticklandii*. Inorganic phosphate and an adenine nucleotide were required for degradation as was a thio compound (dithiopropanol), DPN, Mg^{++}, and, inconsistently, pyridoxal phosphate; ATP^{32} formation from P_i^{32} was observed; arsenate could substitute for inorganic phosphate and adenine nucleotide. Balance studies indicated the reaction:

$$CH_2NH_2COOH + P_i + ADP + R(SH)_2 \xrightarrow{DPN} CH_3COOH + NH_3 + ATP + RSSR.$$

The mechanism of ATP formation is not clear; the authors do not believe it to involve thiol activation, since the reduction of proline to δ-amino valerate by dithiopropanol, also catalyzed by this preparation, did not lead to observable phosphorylation.

Campbell (260) has observed oxidative degradation of glycine by a *Pseudomonas* in which glycine is oxidized to glyoxylic acid and NH_3. Glyoxylate is then oxidized in the presence of thiamine pyrophosphate and Mg^{++} or Mn^{++} to formate and CO_2. Unlike the degradation of glycine in liver, and contrary to expectation, formate arises from glycine carboxyl and CO_2 from the glycine α-carbon. Another degradative pathway for glycine which requires thiamine pyrophosphate and Mg^{++} has been observed by Krakow & Barkulis (261) in extracts of *E. coli* which convert glyoxylate anaerobically to CO_2; hydroxypyruvate is apparently an intermediate. The authors suggest an initial decarboxylation of glyoxylate to form a C_1 fragment at the formaldehyde oxidation level, which then reacts with another molecule of glyoxylate to give hydroxypyruvate or its aldehydic isomer. Flipse has studied glycine metabolism in bovine spermatozoa (262); apparently the pathways involved are similar to those observed in other mammalian tissues. A cell-free extract of a *Pseudomonas* was observed by Campbell (149) to catalyze transamination to glyoxylic acid from glutamate, aspartate, alanine, glutamine, and asparagine. In the course of transamination from the amides, ammonia was formed, suggesting a similarity to liver glutamine transaminase-deaminase. Whether aspartate and alanine transaminate directly, or whether the reaction is mediated through traces of glutamate, is not clearly estab-

lished. Should this reaction, like those of other transaminases, prove reversible, it would provide another pathway, in addition to glycine oxidase, for forming glyoxylate from glycine.

Despite the multiplicity of pathways available to glycine in the mammal (263), none can account satisfactorily for both the net conversion of finite amounts of glycine to CO_2 and water, and, at the same time, the appearance in the phlorhizinized dog of glucose in an amount equivalent to both glycine carbon atoms. The fates of glyoxylate are obscure; the succinate-glycine cycle (264, 265), while providing an efficient pathway for glycine oxidation, requires the loss of one-half of the carbons as CO_2 in the course of generating a C_1 unit which may be condensed to another glycine to form serine, and then carbohydrate. Perhaps additional studies of the origin of carbohydrate from doubly labeled glycine are needed. Nor do any of the pathways observed account for the specific dynamic action of glycine, in which more calories appear as heat than can be accounted for by the combustion of the molecule.

Although the pathway of glycine to carbohydrate is not fully understood, serine dehydrase provides a ready mechanism for the conversion of serine to pyruvate and ammonia. Metzler & Snell (266) had proposed a mechanism for this reaction which involved Schiff's base formation with pyridoxal phosphate, dehydration of the complex to an acrylic acid derivative, and hydrolysis to yield pyruvate, NH_3, and regenerated pyridoxal phosphate. Sayre & Greenberg (267) have achieved considerable purification of serine dehydrase from sheep liver, and have separated it from threonine dehydrase. The serine enzyme was pyridoxal phosphate-dependent; AMP and glutathione were required, but no metal dependence was demonstrated. A serine deaminase in *E. coli* which produced NH_3 faster than pyruvate was observed by Lenti & Grillo (268). These observations are difficult to reconcile with free amino acrylate (269), or its pyridoxal phosphate conjugate (266), as an intermediate.

Metabolism of Methyl Groups

Biogenesis and fate of methyl groups.—It is known that the carbon of C_1 compounds (formate, serine β-carbon, etc.), can appear in methyl groups; conversely, degradation of C^{14} methyl groups can lead to labeling of compounds such as serine, presumably via the C_1 "pool." Nakao & Greenberg (270) and Doctor *et al.* (271) have studied the cofactor requirements for incorporation of serine β-carbon into methionine-methyl by extracts of sheep and chicken liver, respectively. Both laboratories agree that homocysteine, Mg^{++}, DPN, and ATP are required. Pteroyl glutamic acid was needed for the reaction in chicken liver (271); the authors did not describe the effects of other folic acid derivatives, but their preparation was capable of incorporating serine β-carbon into citrovorum factor. In sheep liver (270), leucovorin or THFA stimulated incorporation of formate-C^{14}, but not serine-3-C^{14}, into methionine; THFA markedly inhibited serine incorporation. Deoxypyridoxine inhibited incorporation of both serine and formate, and pyridoxal phosphate

reversed the inhibition. In view of the crudity of the systems employed, it is difficult to assign a precise mechanism to the above observations; indeed, Nakao & Greenberg suggest that transfer of serine-C_3 to methionine by cleavage of a cystathionine-like compound might provide an alternate, non-folic acid, pathway for this transfer. Bregoff & Delwiche have studied formation of the carbon chain and methyl groups of choline and betaine from various labeled precursors in leaf disks of *Beta vulgaris* (272). In general, the observations were consistent with the established mammalian pathway; however, formate was a poor precursor of the carbon chain of betaine and choline, possibly by reason of lack of equilibration with formyl folic acid derivatives. The higher specific activity of choline as compared to that of betaine suggested that the former was first formed in the metabolic chain.

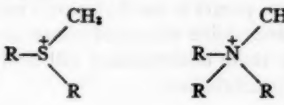
Mackenzie & Abeles (273), using a mitochondrial preparation, have shown that the sarcosine methyl group enters the β-carbon of serine at the formaldehyde oxidation level. Using sarcosine, completely methyl-deuterated, they demonstrated that no transient loss of hydrogen occurred as would have been necessitated had formate been an intermediate. This laboratory has also investigated the binding sites of sarcosine oxidase by means of competitive inhibition analysis (274).

Transmethylation.—Previous studies of Cantoni (275, 276) had demonstrated formation of S-adenosyl methionine (AME) from methionine and ATP; three moles of inorganic phosphate were formed per mole of ATP utilized. Cantoni & Durell (277) have now prepared methionine-activating enzyme free of pyrophosphatase; with this preparation, AME synthesis was accelerated by crystalline yeast pyrophosphatase and inhibited by pyrophosphate. Labeling experiments with ARP—P^{32}—P^{32} and ARP^{32}—P—P indicated that the activation reaction could be formulated as:

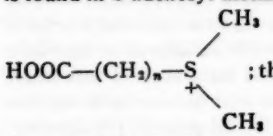
$$\text{L-methionine} + \text{AR—P*—P*—P} \rightarrow \text{AME} + \text{P*—P}_i\text{*} + \text{P}_i.$$

Du Vigneaud's demonstration, in 1947, that methionine methyl groups were transferred intact into creatine and choline, depended upon use of "doubly labeled" methionine prepared by mixing R—$C^{12}D_3$ and R—$C^{14}H_3$. However, Rachele *et al.* (278) observed that —$C^{12}D_3$ groups were metabolized more slowly than $C^{14}H_3$ groups, and suggested that this isotope effect could have led to misinterpretation of the 1947 data. Du Vigneaud, Rachele & White (279) have therefore repeated the prior experiments with methionine labeled with both C^{14} and deuterium in the same molecule; the results obtained were identical with those of the 1947 experiments, and rigorously confirmed the transfer of intact methyl groups from methionine to acceptors.

Cantoni has postulated that an "active" methyl group is one which is part of an "-onium" grouping; i.e.,



This structural feature appears necessary for methylation of an acceptor and is found in S-adenosyl methionine, betaine, and the methyl thetins—



; the latter compounds can therefore serve as methyl donor models. A related compound, S-methyl methionine, has been found in a variety of plants. Schlenk & De Palma (280) have reported that this compound, in the presence of homocysteine and an undefined preparation of *T. utilis*, readily yielded methionine according to the reaction S-methyl methionine+homocysteine→2 methionine; betaine, dimethylacetothetin, and dimethylpropiothetin were inactive as methyl donors. The reaction S-adenosyl methionine+homocysteine→S-adenosyl homocysteine+methionine proceeded slowly, and only in living cells. Maw (281) has reported partial purification of a dimethylthetin-homocysteine transmethylase from rat liver; an enzyme from horse liver which catalyzes this reaction has been obtained by Durell & Anderson (282). The latter preparation, in the presence of glutathione may be homogeneous, but appears to polymerize rapidly in its absence. Thoai & Robin (283) have now observed methionine formation from homocysteine in the liver of a variety of species, including invertebrates, and have obtained a preparation 50 times as active as the original horse liver. McRorie & Carlson (284) have observed that a "leaky" *Neurospora* mutant produced a material which supported the growth of certain mutants which respond to methionine but not to homocysteine, and is therefore an intermediate between these compounds in methionine biosynthesis. The accumulated compound contained an amino group and a thio ether linkage which could be oxidized to an active sulfoxide and an inactive sulfone; it is not identical with S-hydroxymethyl homocysteine or *m*-thiazane-4-carboxylic acid. It is not clear whether this intermediate relates to transmethylation or methyl group neogenesis. Strack & Friedel (285), studying methionine formation from homocysteine and betaine or sarcosine, obtained kinetic data which they suggested was consistent with the formation of a homocysteine-formaldehyde complex which is then reduced to methionine.

Ericson *et al.* have been able to separate apo-enzyme of liver betaine-homocysteine transmethylase from a heat stable cofactor (286, 287); liver homogenates of vitamin B_{12}-deficient rats were low in enzyme activity which was restored to normal by addition of cofactor, the latter containing no detectable vitamin B_{12} derivative. However, in the absence of methyl donors, methionine formation from homocysteine and ATP was completely dependent upon vitamin B_{12} added *in vitro*. Thus, the authors concluded that vitamin B_{12} was active at two points in methyl group metabolism: formation of cofactor of betaine-homocysteine transmethylase and synthesis of methyl groups *de novo*. Perhaps these observations will lead to clarification of the role of this vitamin in C_1 metabolism.

Sulfur-containing Amino Acids

The metabolism of sulfur-containing compounds has been recently reviewed in extenso (190). Although the major steps in the degradative metabolism of cystine seem to have been established, almost nothing is known of the individual responsible enzymes. A system which catalyzes oxidation of cysteine to cystine by DPN has been partially purified (191). Reaction was markedly inhibited by hydroxylamine and accelerated by hypoxanthine, which also overcame the hydroxylamine inhibition. The same preparation also reduced DPN with dimercaptolipoate; however, this reaction was not hydroxylamine-sensitive and therefore appeared to employ a separate enzyme. Awapara & Doctor (192) observed a slow formation of cysteine sulfinic acid (CSA) from cysteine-S^{35} by a washed rat liver residue; maximal activity required the presence of TPN, ATP, and Mg^{++}. Although the rate of reaction was discouragingly slow for the purposes of enzyme purification, the authors calculated that it was sufficient to account for all of the sulfate excreted by the rat. Singer & Kearney (193) have found that, in extracts of rat liver mitochondria acetone powder, CSA is degraded by initial transamination with α-ketoglutarate to β-sulfinylpyruvate which then desulfinates to SO_2 and pyruvate; this pathway has previously been described for rabbit liver (194) and *Proteus vulgaris* (195). The transamination was catalyzed by highly purified glutamic-oxalacetic transaminase. In addition, a second pathway was observed in which CSA was converted to pyruvate, $SO_4^=$, and NH_3 in the absence of α-ketoglutarate (but in the presence of DPN); this suggests that the initial step may be dehydrogenation of CSA to β-sulfinylpyruvate and NH_3 by a DPN-linked enzyme. Chapeville *et al.* (196) demonstrated the fixation of $S^{35}O_3^=$ into cystine by the rabbit *in vivo*. These authors had previously (197) observed incorporation of $S^{35}O_3^=$ into CSA by rabbit kidney preparations and, thus, suggest that the pathway cysteine$\rightarrow$CSA$\rightarrow SO_3^=$ is biologically reversible. In contrast, other workers were unable to demonstrate kinetic reversibility of the desulfination of CSA in the presence of ATP and phosphoenolpyruvate by extracts of *E. coli* which efficiently catalyzed the desulfination reactions, although *E. coli* efficiently employs CSA and sulfite for growth in media otherwise sulfur-deficient (198). It seems unlikely therefore that the SO_2 fixation observed by Chapeville & Fromageot is catalyzed by the enzyme responsible for desulfination of sulfinyl-pyruvate during the usual desulfination series of reactions.

Although various studies in recent years had indicated that taurine formation results from the decarboxylation of CSA, with oxidation of the resultant hypotaurine, Awapara (190) has noted that hypotaurine oxidation *in vivo* is relatively slow and that taurine is formed more rapidly from CSA than from hypotaurine. Clearly, the pathway CSA$\rightarrow$cysteic acid$\rightarrow$taurine seems to be operative. That cysteic acid and CSA are readily decarboxylated by glutamic decarboxylase of rat brain has been reported both by Davison (199) and by Bergeret *et al.* (200). These laboratories also report the exist-

ence in rat (199) and rabbit (200) liver of an enzyme which decarboxylates CSA and cysteate, but not glutamate, and which is activated by pyridoxal phosphate and sulfhydryl reagents. Davison has noted that since lysine, DOPA, and diaminopimelic decarboxylases all require pyridoxal phosphate and sulfhydryl activation as well, these may be general requirements for amino acid decarboxylases. Bergeret & Chatagner have also noted that vitamin B_6-deficient rats secreted taurine in bile only 20 per cent less than normal, and have considered the possibility that there may be an alternate path to taurine which does not require the participation of a pyridoxal phosphate-dependent enzyme (201). Portman & Mann (202) administered taurine-S^{35} to rats and were unable to find any evidence of further metabolism of this compound. Awapara (203) has reported the taurine concentration of various organs of the rat, rabbit, guinea pig, pig, and sheep and the effects of fasting and removal of various organs upon these concentrations in the tissues of the rat. In a preliminary report Machlin (204) stated that after administration of taurine-S^{35} to young chicks, the bulk of the administered material was accounted for as taurine but a significant fraction of the S^{35} was located in an unidentified compound which was not sulfate, cysteate, CSA, or cystine. Eldjarn & Pihl (190) found that cysteamine-S^{35}, only known to be derived from cysteine by way of pantetheine, is rapidly oxidized in liver to hypotaurine which gives rise to sulfate more rapidly than it does to taurine. The metabolic significance of cystamine and its disulfoxide have been thoroughly reviewed by Cavallini (190). A new organic synthesis of hypotaurine, reported by Bricas *et al.* (205), should be of considerable help to workers in this field. Machlin *et al.* have described the partition in the developing embryo of the sulfur of cysteine-S^{35} and methionine-S^{35} among taurine, sulfate, methionine, and cystine (206).

Sorbo (207) has investigated the reactions leading to thiosulfate formation in liver and observed that various heme compounds, including hemin, cytochrome-*c*, and denatured hemoglobin, catalyzed oxidation of H_2S to thiosulfate, and also observed that liver homogenates quantitatively converted mercaptopyruvate to thiosulfate in the presence of sulfite. The combination of CSA and mercaptopyruvate, presumably derived from transamination of cysteine, was observed to yield a new compound—alanine thiosulfonate. Hypotaurine yielded the analogous β-aminoethane thiosulfonate. Fridovich & Handler (208) described a partially purified enzyme preparation from rat liver which catalyzed the aerobic oxidation of sulfite to sulfate and the anaerobic reduction of methylene blue and other dyes by sulfite. The preparation was inactivated by dialysis and reactivated by a thermostable factor from liver which was isolated and identified as hypoxanthine. The function of hypoxanthine in this system was not established, but it is of interest that this compound has been reported to be essential for the operation of a cysteine dehydrogenase of liver (191), for the desaturation of fatty acids by a crude enzyme system from rat liver (209), and for the opera-

tion of an incompletely characterized phospholipide dehydrogenase of liver (210, 211). The initial step in the operation of this sulfite oxidase has been identified (212) as a reaction between sulfite and lipoate with the formation of lipoate hemithiosulfonate which is then hydrolyzed to sulfate and reduced lipoate. The hypoxanthine requirement is presumed to relate to the subsequent oxidation of dimercaptolipoate.

Chatagner & Saurette-Ignazi (213) have observed that the appearance of ammonia follows, rather than precedes, the appearance of H_2S during the action of crude cysteine desulfhydrase preparations from rat liver and *E. coli;* they suggest that cysteine desulfhydrase may be an enzyme complex which catalyzes the following series of reactions:

(*a*) cysteine + α-ketoglutarate → β-mercaptopyruvate + glutamate
(*b*) β-mercaptopyruvate → S + pyruvate
(*c*) S + RH → H_2S
(*d*) glutamate → NH_3 + α-ketoglutarate

Small quantities of sulfur were observed to appear early in the reaction. However, the suggestion is difficult to reconcile with the observations of Dietrich & Borries (214) who found that deoxypyridoxine phosphate markedly inhibited glutamic-aspartic transaminase and DOPA decarboxylase and that the activity of both these enzymes was markedly reduced in B_6-deficient rats, whereas neither the antimetabolite nor pyridoxine deficiency affected the cysteine sulfhydrase activity of rat liver. A preparation of cysteine desulfhydrase was found to be activated by pyridoxal phosphate but not inhibited by deoxypyridoxal phosphate at a concentration which markedly inhibits tyrosine decarboxylase and pig heart transaminase. The author concludes that the antimetabolite has little affinity for cysteine desulfhydrase, but, if the formulation of Chatagner *et al.* were correct, inhibition of cysteine desulfhydrase would seem likely. Of interest in this connection is the observation of Artman *et al.* (215) that dihydrostreptomycin at low concentrations noncompetitively and irreversibly inhibited the cysteine desulfhydrase of cell-free extracts of *E. coli.*

Mills & Wood (216), after administration of iodobenzene-I^{131} to rats, isolated iodophenyl cysteine from liver, kidney, and intestine but could not find this material in urine, thus establishing that acetylation is the final step in mercapturic acid synthesis. No I^{131} was found in tissue protein under these circumstances.

Racker (217) has partially purified a liver glutathione-homocystine transhydrogenase which catalyzes the reaction GSH+homocystine→GSSG+homocysteine. The system can be coupled with glutathione reductase, thus providing a mechanism for reduction of homocystine by reduced pyridine nucleotides. A new and specific color test for homocysteine has been described by Pasieka & Morgan (218).

Elder & Mortensen (219) demonstrated incorporation *in vitro* of labeled glutamate and glycine into the glutathione of both rat and duck erythrocytes.

When glycine-C^{14} was given to the rat *in vivo* (220), erythrocyte glutathione activity reached a maximum at about 1 hr. and then declined exponentially. The half time was about 65 hr. At all times the ratio of glycine to cystine to glutamate activity remained constant, indicating failure of reutilization of the amino acids; this is consistent with the concept that the turnover of glutathione represents synthesis *de novo*. Glutathione turnover in both brain and liver is considerably more rapid than in the erythrocyte, proceeding with a half life of about 4 hr. in liver and 7 hr. in brain (221). As would be expected from much previous experience, glycine-C^{14} administered intraperitoneally was not found in brain glutathione; incorporation was readily achieved after intracisternal injection.

The isolation of S-methyl L-cysteine from bean seeds was announced by Thompson *et al.* (222); the corresponding sulfoxide had previously been found in crucifers (223, 224, 225). Wolff, Black & Downey have reported formation of S-methyl cysteine from methyl mercaptan and serine in the presence of an eightfold purified enzyme from yeast (226). No reaction was observed with homoserine in place of serine nor with H_2S in place of methyl mercaptan, thus eliminating the possibility that the enzyme in question is cysteine desulfhydrase. The mechanism of this reaction and its metabolic significance remain obscure.

Arginine

The biosynthesis of ornithine and arginine and properties of arginase are described elsewhere in this review. An interesting new pathway of arginine metabolism has been observed in the laboratory of Roche. *Streptomyces griseus*, adapted to arginine, exhibits an enzyme system which converts arginine to guanidobutyramide which in turn hydrolyzes to guanidobutyrate plus ammonia (119, 120, 121). Evidence was presented that the α-amino nitrogen remains fixed to the carbon chain during formation of the amide; the mechanism of this unusual reaction is unknown. Extracts of a series of insects were also found to form small quantities of guanidobutyramide from arginine, although the dominant pathway was consecutive formation of guanido-α-ketovaleric acid, guanidobutyraldehyde, and guanidobutyrate (122, 123). The subsequent fate of guanidobutyrate remains unknown. Ennor *et al.* (124) have described a method for the preparation of phosphoarginine from crayfish muscle which yields almost 20 times as much material as do older procedures.

C_5-Amino Acids

The interconversions among the C_5-amino acids remain a subject of much interest. The generally held notion that these compounds find their origin in glutamic acid whose carbon chain is derived from carbohydrate has been challenged by two different groups. Swick & Handa (125) have followed the path of $C^{14}O_2$ into the free amino acids of rat liver, while Vavra & Johnson (126) have performed similar studies with bakers' yeast. Both groups con-

clude that arginine, and perhaps proline, must have some precursor other than, or in addition to, glutamic acid. The data of the former group also suggest a feedback mechanism since the amount of carbon fixed in the C_5-amino acids of rat liver was two or three times as high when these amino acids were omitted from the diet.

Vogel & Bonner have achieved partial purification from *E. coli* of the N-α-acetylornithinase necessary for ornithine production (starting from glutamate). The preparation was activated by Co^{++} and GSH and of a series of other acetylamino acids, slowly hydrolyzed only acetylmethionine (127). Smith & Greenberg have partially purified an enzyme from rat liver which catalyzes the reduction of pyrolline-5-carboxylic acid to proline by DPNH (128); a similar enzyme had been reported in *N. crassa*. Proline oxidase has been found to be located almost exclusively in mitochondria where it is markedly inhibited by Ca^{++} (129). Reduction of proline to δ-aminovaleric acid by a *Clostridium* has been reported by Stadtman (130). Various dithiols, including lipoic acid, served as reducing agents in unfractionated extracts which also required DPN, Mg^{++}, and pyridoxal phosphate. Vogel (131), using ornithine-C^{14}, found that whereas this amino acid is converted to proline which is used for protein synthesis, it is a minor metabolic pathway and suggests that the major path from glutamate does not involve ornithine as an intermediate. Three preliminary reports have appeared concerning the fate of hydroxyproline. D-amino acid oxidase was found to oxidize D-hydroxyproline to pyrolle α-carboxylic acid, probably by way of an intermediate, Δ'-pyrolline-4-hydroxy-2-carboxylic acid (501). Adams (132) found that *Pseudomonas*, adapted to hydroxyproline, converted it to L-glutamate; these studies did not establish the intermediate pathway. Heck *et al.* (133) gave hydroxyproline-C^{14} to intact rats and found radioactivity in the alanine of liver protein as well as lesser activity in aspartate and glutamate. Ravel & Shive (134) reported a series of complex interrelationships among the C_5-amino acids with respect to the growth of *S. lactis* 8034. Biserte *et al.* (135) have isolated from normal human urine homopantotheine in which γ-aminobutyrate replaces β-alanine. A new pathway of glutamate decomposition in *Cl. tetanomorphum* (136) has been indicated by the finding of appreciable quantities of 2-methylfumarate during glutamate fermentation.

Of considerable interest is the observation by Tallan, Moore & Stein (137) that as much as 100 mg. per cent of N-acetyl-L-aspartate occurs in rat and cat brain. The compound was not found in kidney, liver, muscle, or urine of the cat. Its function is unknown and the compound is noteworthy in that it is the first simple acetyl amino acid isolated from animal tissue (137). The aspartase of *B. cadaveris* has been obtained in highly purified form (138) and some of its properties have been described.

Homoserine and Threonine

Biosynthesis of threonine in a number of microorganisms appears to involve the steps: aspartate→β-aspartyl phosphate→aspartic β-semialdehyde

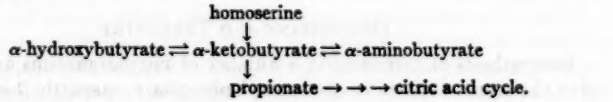
→homoserine→threonine (288, 289). Condensation of glycine with acetaldehyde may furnish an additional pathway to threonine (290, 295). Nisman *et al.* (291) have observed an ATP and pyridoxal phosphate requirement for the conversion of homoserine to threonine by *E. coli;* in the presence of ATP, homoserine, and hydroxylamine, a hydroxamic acid was formed, suggesting a carboxyl-activated intermediate; pyridoxal phosphate appeared to be required for a step beyond the activation of homoserine.

Watanabe & Shimura (292, 293, 294) have also observed an ATP requirement for synthesis of threonine from homoserine by yeast extract. The enzymes catalyzing this conversion were separated into two fractions; one of these appeared to catalyze synthesis of O-phosphohomoserine, which was isolated and partially characterized. The other fraction converted phosphohomoserine to threonine; release of inorganic phosphate was more rapid than threonine formation, suggesting the existence of additional intermediates.

A brief communication from Karsek & Greenberg (295) notes the partial purification of sheep liver enzymes which catalyze a "threonine aldolase" reaction, with production of glycine and acetaldehyde. The preparation was most active toward L-allothreonine and least active toward L-threonine; allothreonine, but not threonine, cleavage was markedly stimulated by pyridoxal phosphate. A compound tentatively identified as threonine was synthesized from acetaldehyde and glycine upon incubation with the enzyme preparation. Whether the steps in the "threonine aldolase" reaction are analogous to the "serine aldolase" reaction sequence should be of considerable interest. Since the initial step in the latter sequence is formation of a serine-THFA complex, followed by a pyridoxal phosphate-mediated cleavage to glycine and hydroxymethyl-THFA, an analogous sequence for "threonine aldolase" would involve formation of threonine-THFA and cleavage of the latter to acetaldehyde-THFA and glycine.

A threonine dehydrase from sheep liver has been purified 620-fold by Sayre & Greenberg (267). Aged preparations of this enzyme were stimulated by pyridoxal phosphate; AMP and glutathione were also stimulatory. Umbarger & Brown (296) have observed L- and D-threonine dehydrases in *E. coli;* both enzymes were stimulated by pyridoxal phosphate.

Matsuo *et al.* (297) have observed that incubation of rat liver homogenate with DL-homoserine-2-C^{14} led to labeling of α-keto butyrate, α-hydroxy butyrate, α-amino butyrate, and propionate; C^{14} was not detected in homocysteine, methionine, threonine, alanine, or α-keto-γ-hydroxy butyrate. An ammonium sulfate fraction yielded C^{14} α-ketobutyrate from homoserine as the sole radioactive product. On the basis of these data, the authors propose the following scheme:



They suggest that the initiating reaction is analogous to serine and threonine dehydration. The observation that C^{14} homoserine does not produce labeled homocysteine suggests that the former compound, although formed by cystathionine cleavage, cannot condense with cysteine to form cystathionine. Studies of Kisliuk *et al.* (298) on the fate of the methionine carbon chain in the intact rat indicate that the major pathway of methionine carbon, presumably via cystathionine, is identical with the degradative path of homoserine, rather than by primary formation of the α-keto acid of methionine. Since homoserine is available to the mammal, and its only metabolic pathway appears to be degradative, it appears that the metabolic block in threonine synthesis in "threonineless" mammals, such as man and the rat, lies between homoserine and threonine; the precise localization of the "block" would be of interest. In addition the dietary essentiality of threonine, despite the observation that mammalian tissues can form threonine by acetaldehyde-glycine condensation (295, 299), suggests again that free acetaldehyde is not a normal metabolite.

Valine, Leucine, and Isoleucine

Biosynthesis of valine, leucine, and isoleucine.—2-Ketoisovaleric acid, "keto valine," appears to be an intermediate in both valine and leucine biosynthesis in several microorganisms; this keto acid may either be aminated to form valine, or condensed with an acetate unit, with loss of CO_2, to form "keto leucine." The pathway of ketovaline biosynthesis appears to involve condensation of two molecules of pyruvate with an intramolecular rearrangement (328 to 331).

Strassman *et al.* (332) have extended their previous studies (333) of leucine biosynthesis in *T. utilis*. Growth of yeast with tracer quantities of C^{14}-acetate and lactate indicated that carbons 1 and 2 of leucine arose from acetate carboxyl and methyl carbons, respectively; the other four carbons originated from the isobutyl portion of valine. The authors have proposed a mechanism involving condensation of α-ketoisovalerate with acetyl-CoA to yield, ultimately, 2-keto isocaproic acid by a series of reactions analogous to formation of glutamate from oxalacetate. These observations confirm similar conclusions of Abelson & Vogel (301, 334) based upon isotope competition studies; the mechanism resembles one proposed by Strassman & Weinhouse (51, 300) for the origin of α-amino adipic acid in lysine biosynthesis by yeast.

Although abundant evidence exists that threonine is a precursor of isoleucine in *E. coli* (335) and *Neurospora*, probably through α-keto-isobutyrate and the α,β-dihydroxy analogue of isoleucine (336), the pathway of biosynthesis of this amino acid remains an enigma. Little is known beyond the fact that carbons 1, 2, 3, and 4 of threonine form, respectively, carbons 1, 2, 5, and 6 of isoleucine, and that C-3 and the methyl group can be derived from acetate methyl. Adelberg (336) has presented a hypothetical scheme of isoleucine biosynthesis to account for this isotope distribution.

Umbarger (337) has observed that addition of isoleucine to *E. coli* strongly inhibits the synthesis of this amino acid from threonine, thereby providing a "feed-back" mechanism which controls the amount of isoleucine synthesized; the locus of inhibition appears to be threonine dehydrase, which is competitively inhibited by isoleucine. Broquist & Stiffey (54) have observed inhibition of growth of *T. cremoris* by lipoic acid analogues, which is noncompetitively reversed by valine plus isoleucine, and competitively reversed by fatty acids, but not by lipoic acid. The authors suggest that the analogues interfere with biosynthesis of isoleucine and valine.

Degradation of branched chain amino acids.—The pathways of degradation of the branched chain amino acids have been intensively studied in Coon's laboratory (338), The degradation of leucine appears to be initiated by formation of the corresponding keto acid, which is then decarboxylated with formation of isovaleryl CoA (338), in a reaction formally analogous to formation of acetyl CoA from pyruvate. The degradation of isovaleryl CoA then proceeds by the following steps (339, 340):

(*a*) isovaleryl CoA $\rightleftharpoons$ β-methyl-crotonyl CoA (+2H)
(*b*) β-methyl-crotonyl CoA + $H_2O \rightleftharpoons$ β-hydroxyisovaleryl CoA
(*c*) β-hydroxyisovaleryl CoA + $CO_2 \overset{ATP}{\rightleftharpoons}$ β-hydroxy-methyl-glutaryl CoA
(*d*) β-hydroxy-methyl-glutaryl CoA $\rightleftharpoons$ acetoacetate + acetyl CoA

isovaleryl CoA + H_2O + $CO_2 \rightarrow$ acetoacetate + acetyl CoA.

These observations are in accord with the known formation of 1.5 moles of acetoacetate per mole of leucine. Bacchawat *et al.* (340) have obtained a heart enzyme fraction which forms "active CO_2," possibly adenosine phosphoryl carbonate, from CO_2 and ATP; a second enzyme fraction, in the presence of β-hydroxyisovaleryl CoA and the product of fraction 1, carboxylates β-hydroxyisovaleryl CoA to β-hydroxy-methyl glutaryl CoA.

The initial steps in isoleucine degradation resemble those of leucine, with formation of α-methyl butyryl CoA (338); the latter compound then appears to be degraded as follows (341):

(*a*) α-methyl-butyryl CoA $\rightleftharpoons$ tiglyl CoA (+2H)
(*b*) tiglyl CoA + $H_2O \rightleftharpoons$ α-methyl-β-hydroxy-butyryl CoA
(*c*) α-methyl-β-hydroxy-butyryl CoA + $DPN^+ \rightleftharpoons$ α-methyl-acetoacetyl CoA + DPNH + H^+
(*d*) α-methyl-acetoacetyl CoA + CoA $\rightleftharpoons$ acetyl CoA + propionyl CoA

α-methyl-butyryl CoA $\rightarrow$ acetyl CoA + propionyl CoA.

The authors suggest (341) that these data can account for the weakly ketogenic and glucogenic action of isoleucine; reversal of reaction (*d*) is suggested as a possible mechanism for synthesis of branched chain compounds.

The degradation of valine is not as clearly understood as that of the other branched chain amino acids (338); isobutyryl CoA and β-hydroxyisobutyryl CoA appear to be intermediates. Robinson (342) has observed an enzyme in microorganisms and animal tissues which, in the presence of DPN, converts β-hydroxyisobutyrate (but not its CoA derivative) to α-

methyl malonaldehyde. He suggests that CoA is removed from the hydroxy acid prior to oxidation; the absence of CoA from the resulting aldehyde should permit ready loss of the carboxyl as CO_2. This would leave a three-carbon fragment which, perhaps as propionate, could form glucose, in accord with the established observation that three of the five valine atoms form glucose in the phlorhizinized dog.

Lysine

Lysine biosynthesis appears to proceed by two different mechanisms: in yeast and *Neurospora*, α-amino adipic acid is a precursor; in *E. coli*, diaminopimelic acid (DAP). The α-amino adipic pathway appears to be a variant of a synthetic mechanism shared by glutamate and leucine, i.e., addition of a two-carbon unit to an α-keto acid, followed by reactions analogous to the citric acid cycle (51). Thus, condensation of acetyl CoA with oxalacetate yields, ultimately, α-ketoglutarate, and similar condensation with α-ketoisovalerate yields the keto acid of leucine. In synthesis of the α-amino adipic acid, α-keto-glutarate is the acetyl acceptor (300). Although the yeast and *Neurospora* pathways are essentially similar, the C_2 fragment does not appear to be derivable from acetate in the latter organism (301).

The DAP pathway of lysine biosynthesis, found in *E. coli*, is less well understood (302, 303); this acid appears to arise, by unknown reactions, from aspartate and is decarboxylated to lysine. The apparent intervention of threonine in certain *E. coli* mutants may be referable to its sparing action on aspartate, from which it is derived via homoserine. Strassman *et al.* (304) have grown a wild *E. coli* strain in a medium of C^{12}-glucose and variously labelled acetate and lactate; the distribution of isotope in degraded lysine was incompatible with the biosynthetic pathway for α-amino adipic acid, confirming the nonidentity of the *E. coli* pathway with that in yeast and *Neurospora*. Gilvarg (305) observed that aspartate was superior to ammonia as a nitrogen source for DAP synthesis in the presence of glucose by *E. coli* mutant 26-26, which lacks DAP decarboxylase. Homoserine and threonine were less effective than aspartate or even NH_3; cell-free extracts capable of synthesizing DAP were obtained; pyruvate, aspartate, glutamate, Mg^{++}, ATP, DPN, and TPN were found to be stimulatory. Rhuland & Bannister (306) have obtained a material, apparently succinate, from supernatants of strain 26-26, which stimulates the growth of DAP-requiring strain 81-29. These workers, like Gilvarg (305), have obtained a cell-free extract of strain 26-26 capable of DAP synthesis; aspartate, succinate, pyruvate, TPN, and ATP stimulated this synthesis.

Readers are referred to the excellent survey of Work (302), which includes detailed discussion of lysine degradation in various species.

Histidine

Biosynthesis of histidine.—Although the steps from imidazole glycerol phosphate (IGP) to histidine (IGP→imidazole acetol phosphate→histidinol

phosphate→histidinol→?histidinal→histidine) appear fairly well established, the origin of IGP is but poorly understood (307). Nothing is known of the origin of imidazole-N_3 of histidine; imidazole-C_2 can arise from formate, and Neidle & Waelsch (61) have now shown that N_1 can arise, without dilution with NH_3 or asparagine-N, from glutamine amide-N in *E. coli*. Magasanik *et al.* (59) have shown that *E. coli* mutant HP-1, whose guanine requirement is spared by histidine, could derive C_2 of the imidazole ring exclusively from C_2 of guanine, and that N_1 arose from the guanine amino group, without dilution, from an ammonia pool (60). Thus, the C_2 and N_1 of histidine imidazole appear to derive by transfer, as a unit, of C_2 of guanine with its associated amino group (60); Neidle & Waelsch (61) have independently suggested group transfer from guanine as one possible means of transferring nitrogen from glutamine to histidine-N_1. The observations that glutamine-amide-N and guanine-amino N can each form histidine imidazole-N_1 are not necessarily contradictory, since the mechanism of amination of xanthylic to guanylic acid (59) does not exclude glutamine as an intermediate.

Westley & Ceithaml have concluded, on the basis of inability of certain *E. coli* mutants to utilize short-chain imidazole derivatives, that the five-carbon chain of histidine is established prior to formation of the imidazole ring (308). Further studies (309) on the origin of histidinol carbons from glucose-1-C^{14} and -6-C^{14} in *E. coli* indicate that the five-carbon chain is derived from condensation of C_2 and C_3 units arising from glycolysis and subsequent oxidation, without participation of the hexose monophosphate shunt. Whether PRPP could be an intermediate, as it is in synthesis of indole glycerol phosphate, cannot be deduced from these studies.

Ames (310) has partially purified the *Neurospora* dehydrase which catalyses the conversion of IGP to imidazole acetol phosphate, and has shown it to be activated by cysteine and Mn^{++}, inhibited by versene, and inactive toward dephosphorylated derivatives. Ames & Horecker have published a detailed study of imidazole acetol phosphate transaminase (147) which converts the latter to histidinol phosphate; pyridoxal phosphate is required, and the enzyme exhibits considerable cross-specificity. No intermediates in the DPN-catalysed conversion of histidinol to histidine have been observed; Adams (311) has synthesized histidinol and shown that a hundred-fold purified enzyme from yeast or *Arthrobacter histidinolovorans* can reduce or oxidize the aldehyde to the alcohol or histidine. One enzyme appears to catalyze both transformations (312).

Histidine degradation.—The degradative pathways of histidine have been reviewed by Tabor (244). Wolf *et al.* (313) have conducted a thorough survey of the histidine metabolites excreted by rats injected with DL-histidine-α-C^{14}. Although their studies suggest that the urocanic acid pathway of histidine degradation may not have the same importance in the rat as in microorganisms, factors such as pool dilution and use of racemic histidine render interpretation difficult; excretion of methylated histidine derivatives was ob-

served. Cowgill reports (314) that imidazole was metabolized to the extent of 25 to 55 per cent by the rat; 1-methylimidazole was not demethylated. The 1- and 3-methyl histidines were excreted mostly unchanged by chicks, rats, rabbits, and frogs (315); only 1 per cent of C^{14}-methyl appeared as CO_2.

Karjala *et al.* have isolated 1-methylimidazole-4- and -5-acetic acid (316) and imidazole acetic acid riboside (317) from urine of mice fed large quantities of histamine. When physiological quantities of histamine are administered, only N_3 of the imidazole ring is methylated (318); Schayer's previously described "histamine-metabolizing enzyme II" appears to be a combination of a methylating enzyme and an oxidizing enzyme (probably not identical with monoamine or diamine oxidase) which oxidizes N_3-methyl histamine to the corresponding methylimidazole acetic acid (318, 319). Schayer reports that free mast cells of rat peritoneal fluid (320, 321), as well as rabbit platelets (322), contain a soluble histidine decarboxylase and bind histamine. Preincubation of rat mast cells with nonisotopic histamine had no effect upon the subsequent formation and binding of histamine from C^{14}-histidine, suggesting that exogenous histamine cannot enter the bound condition in free mast cells (321).

Other histidine derivatives.—McManus (323) fed methionine methyl-C^{14} to rats and concluded that methionine methyl was directly transferred to carnosine to form anserine. Melville *et al.* (324) report that ergothionine, which is not made by animals but is found in plants, was synthesized by nine species of fungi, but not by twelve bacteria tested; in *Neurospora*, the carbon and nitrogen skeleton is derived from histidine, the methyl groups from methionine, and the sulfur from cysteine or methionine (325); thiolhistidine does not appear to serve as a precursor. Ban & Stowell (326) describe production of ergothionine by the ergot fungus, *Claviceps purpura*, in defined culture. The compound can be found in mycelial extract, but not in culture filtrate. Goodall (327) reports that synthetic carnosine phosphate mimics creatine phosphate in relaxing glycerol-extracted muscle fiber.

Aromatic Amino Acids

Biosynthesis of the aromatic ring.—It is generally accepetd that shikimic acid (SA) is a precursor of the aromatic rings of phenylalanine, tyrosine, and tryptophan (303). Although sedoheptulose-diphosphate (SDP) can act as precursor of SA, isotopic studies indicate that SDP does not retain its carbon chain intact; Srinivasan *et al.* (343) demonstrated that SDP was active only to the extent of furnishing tetrose and triose; erythrose-4-phosphate plus phosphoenol pyruvate caused formation of dehydro-SA in cell-free preparations of an *E. coli* mutant. Srinivasan *et al.* (344) have extended the studies of Sprinson's laboratory (345, 346, 347) on the origin of the shikimic acid carbons from C^{14}-glucose; the carboxyl, C_1, and C_2 atoms of SA were found to originate from a three-carbon glycolysis intermediate, and the remaining carbons from tetrose phosphate generated via the pentose pathway.

Thus, the isotopic data are consistent with the tetrose plus triose pathway

observed in cell-free extracts (343). This pathway appears to proceed via 2-keto-3-deoxy-7-phospho-D-*gluco*heptonic acid (348); the latter compound has been synthesized, is readily utilized for dehydro-SA formation by *E. coli* mutant 83-24, and may be identical with compound "V" (303), a phosphorylated α-keto acid accumulated by mutant 83-3 and utilized by mutant 83-24 for dehydro-SA formation. Cell-free extracts of the latter mutant have been fractionated; one fraction utilized phosphoenol pyruvate in the presence of erythrose-4-phosphate without dehydro-SA formation, and the other converted the phospho-keto acid to dehydro-SA with release of one mole of P_i.

Biosynthesis of phenylalanine and tyrosine.—Although prephenic acid appears to be on the pathway of aromatic amino acid synthesis between shikimic acid and phenylalanine (303, 349), present evidence does not state whether this hydroxy acid can be directly converted to tyrosine, or whether phenylalanine must first be formed and then hydroxylated. Ghosh (350) has investigated the prephenic acid-to-tyrosine pathway in an *E. coli* mutant blocked in the conversion of prephenic acid to phenylpyruvate; cell-free extracts converted prephenic acid to a material with properties of *p*-hydroxyphenyllactate. Upon addition of cell residue, a tyrosine-like material was formed. Thus, a pathway from prephenic acid to tyrosine not involving phenylpyruvate appears to be available.

Metzenberg & Mitchell (351) have prepared a *Neurospora* triple mutant requiring phenylalanine, tyrosine, and tryptophan by crossing strains which required each of these three alone; this mutant secreted large quantities of prephenic acid into the medium. A single, tryptophan-requiring parent mutant accumulated small quantities of this acid, but the authors, on structural grounds, suggest that prephenic acid accumulation in this mutant represents shifting of equilibria rather than direct derivation of tryptophan from prephenic acid. Barratt *et al.* (352) have attempted to elucidate the nature of the block in an unusual *Neurospora* strain which responds to any of the following: phenylalanine, phenylpyruvic acid, tyrosine, ethyl acetoacetate, leucine, norleucine, tryptophan, indole pyruvic acid, indole, and kynurenine; genetic analysis had indicated that only one locus was responsible for these characteristics. Results with C^{14}-phenylalanine and C^{14}-leucine indicated that these amino acids were not interconvertible, nor did they appear to function through acetoacetate; the nature of the block therefore remains obscure.

Biosynthesis of tryptophan.—Although anthranilic acid is recognized as a tryptophan precursor in a number of organisms, the mechanism of ring closure to form the indole nucleus has, until recently, been unknown. Yanofsky (353, 354) has now described a pathway whereby PRPP furnishes its C_1 and C_2 to form C_2 and C_3 of indole with elimination of the anthranilic acid carboxyl; carbons 3,4, and 5 of PRPP form the side chain. Separate fractions of *E. coli* extract were prepared; fraction A accumulated a compound containing P^{32} from P^{32} RPP, lacking the anthranilic acid carboxyl,

and characterized as indole-3-glycerol phosphate; fraction B converted the latter to indole and triose phosphate (354).

Although it is tempting to consider that the glycerol phosphate side chain may yield the tryptophan side chain directly, in a manner analogous to the formation of histidine from imidazole glycerol phosphate, this fact is by no means established. Yanofsky (354) was unable to substitute indole-3-glycerol phosphate or indole-3-glycerol for anthranilic acid or indole in mutant *E. coli* strains responding to the latter compounds. He suggests that the cells may be impermeable to the phosphorylated derivative, and may be unable to phosphorylate free indole glycerol, and cites mutant *E. coli* strains which appear to accumulate indole-3-glycerol. Should an indole glycerol phosphate pathway indeed be operative, it would provide another source besides serine, for the alanine moiety of tryptophan. It is of interest that in histidine biosynthesis, where the fate of a glycerol phosphate side chain is understood, its origin is obscure; in tryptophan biosynthesis, the origin of this side chain is understood, but its contribution to tryptophan biosynthesis has yet to be established.

Brenner (355) has observed a *Salmonella* mutant which responds to indole and tryptophan, but not to anthranilic acid; this mutant accumulates an uncharacterized intermediate compound "B," which is insoluble in ether, gives a blue color with Bratton-Marshall reagent, which is unaffected by prior acetylation, and is converted to indole by hot dilute alkali. It would be of interest to learn whether these properties correspond with those of indole-3-glycerol phosphate. Gibson *et al.* (356) have studied the cultural conditions for indole synthesis by *E. coli* strain 7-4, which is unable to convert indole to tryptophan.

Tryptophan degradation.—Tryptophan degradation can occur by a variety of pathways; the reader is referred to Mehler's comprehensive review (357) for the context into which to place the current year's contributions. Although 3-hydroxykynurenic acid is recognized as a product of tryptophan degradation, its mode of formation has been poorly understood. De Castro *et al.* (358) have now described a liver mitochondrial system which converts L-kynurenine to 3-hydroxykynurenine. TPNH and O_2 were required, suggesting direct hydroxylation of the ring; unlike nonenzymatic hydroxylation, the present system was specific for L-kynurenine. Another pathway of kynurenine metabolism, catalyzed by kynureninase, is the cleavage to anthranilic acid and alanine; Wiss & Weber (359) have obtained an electrophoretically homogeneous preparation of this enzyme from pork liver; pyridoxine was obtained on hydrolysis, confirming the participation of pyridoxal phosphate in the reaction. The "acyl pyruvase" reaction was not catalyzed by purified kynureninase. Kynurenine transaminase, which catalyzes formation of kynurenic acid from kynurenine, has been partially purified from rat kidney (150) and *Neurospora* (151). Pyridoxal phosphate was required by both preparations; a variety of α-keto acids served as amino group acceptors in the *Neurospora* preparation, which also transaminated 3-hydroxykynure-

nine to xanthurenic acid. The role of pyridoxal phosphate in reactions involving kynureninase and kynurenine transaminase is further confirmed by the increased excretion of kynurenine and 3-hydroxykynurenine observed in patients on isoniazid therapy (360).

The conversion of indole nitrogen to pyridine nitrogen appears to occur by splitting of 3-hydroxyanthranilic acid and cyclization of a presumed intermediate amino aldehyde to quinolinic acid. This intermediate aldehyde has been obtained by Wiss *et al.* (361) from 3-hydroxyanthranilic acid in the presence of a dialyzed extract of pig liver acetone powder. Its structure appears to be 1-amino-4-formyl-buta-1,3-diene dicarboxylic acid, which is consistent with the 3,4 split in the anthranilic acid ring suggested by a number of previous observations, and recently supported by isotopic studies of Henderson & Hankes (362) on carbon distribution in rat urine quinolinic acid after ring-C^{14}-tryptophan administration. However, whether quinolinic acid is the direct precursor of nicotinic acid is not clearly established; further doubt as to the importance of this pathway is raised by studies of Quagliariello & Della Pietra (363) who found that 12-day chick embryos converted 3-hydroxyanthranilic acid to nicotinic acid to a small extent, but failed to convert quinolinic to nicotinic, or 3-hydroxyanthranilic to quinolinic acids. Tabone & Tabone (364) have observed that preparations from *B. megatherium* formed anthranilic acid glucoside from uridine diphosphoglucose and anthranilic acid. Brown & Price (365) found that whereas rats excreted anthranilic acid mostly unchanged and but 10 to 25 per cent as *o*-amino hippurate, humans excreted virtually all of the acid administered, as the glycine conjugate.

The hydroxylation pathway of tryptophan degradation, of importance in serotonin (5-hydroxytryptamine) formation, appears to be of major importance in *Chromobacterium violaceum* (366); 5-hydroxytryptophan has been isolated from culture filtrates, and the hydroxylation reaction appears to be specific for tryptophan. No evidence for the kynurenine pathway was observed in this organism. A role for pyridoxal phosphate in 5-hydroxytryptophan decarboxylase receives further support from the observations of Buxton & Sinclair (165) that impaired kidney decarboxylase activity of rats receiving deoxypyridoxine could be restored by addition *in vitro* of pyridoxal phosphate. Adam & Weiss (367) have identified 5-hydroxytryptamine as a component of scorpion venom.

Brown & Price have studied the excretion pattern of a number of tryptophan metabolites after administration of L-tryptophan to the dog, cat, rat, and man (368) and D-, DL-, acetyl-L- and acetyl-D-tryptophan to man (369). The D-isomer was effectively utilized but produced an excretion pattern distinctly different from that of the L; acetyl-D-tryptophan was inert, and acetyl-L-tryptophan was metabolized more slowly than the free amino acid. Beerstecher & Edmonds (370), in a preliminary report, state that indole serves as a cofactor for conversion of 5-methyl tryptophan to tryptophan by *E. coli*. Katagiri & Hayaishi (371) have reported that β-keto adipic acid, a

product of the "aromatic" pathway of tryptophan degradation, was stoichiometrically converted to succinate and acetyl CoA by extracts of tryptophan-adapted *Pseudomonas* in the presence of catalytic quantities of succinyl CoA and substrate quantities of CoA. A cyclic mechanism, involving formation of β-keto adipyl CoA, thiolysis to produce acetyl CoA and succinyl CoA, and utilization of succinyl CoA to activate another β-ketoadipic acid molecule, was proposed.

Metabolism of phenylalanine and tyrosine.—Mitoma has demonstrated that two separate protein fractions are required for hydroxylation of phenylalanine to tyrosine (372) by rat liver in the presence of DPNH and O_2; despite this observation, attempts to demonstrate two steps in the reaction were unsuccessful, as were efforts to implicate hydrogen peroxide. Dalgliesh & Tabechian (373) have observed that the rate of $C^{14}O_2$ production from uniformly-labelled tyrosine was double that from phenylalanine in the rat, and concluded that phenylalanine hydroxylation is a rate-limiting step.

La Du & Zannoni (374) observed that extracts of dog liver acetone powder which catalyzed oxidation of *p*-hydroxyphenylpyruvate to homogentisate were unable to oxidize 2,5-dihydroxyphenylpyruvic acid, suggesting that this acid was not an intermediate. p-Hydroxyphenylpyruvate oxidation was inhibited by diethyldithiocarbamate, thus localizing the inhibitory action of that compound on tyrosine oxidation; inhibition was partially reversed by Cu^{++}. Further fractionation of liver established a requirement for two enzymes (375), one of which appeared to be catalase (376, 377), whose action is unexplained. Unlike acetone powder extracts, the resolved system was unaffected by ascorbate, but was stimulated by reduced 2,6-dichlorophenolindophenol. Preaddition of substrate to noncatalase fraction B prevented reduced dye stimulation; this observation, as well as inhibition of the system by diethyldithiocarbamate, has led the authors to suggest that reduced dye may be required for reduction of Cu^{++} bound to fraction B.

Pitt & Knox (378) have described an enzyme from hog liver and kidney which catalyzed keto-enol tautomerism of phenyl and *p*-hydroxyphenyl pyruvate; *m*-hydroxy- and methoxy-phenylpyruvates were less active, and indole and imidazole pyruvates were virtually inert. No action was observed on aliphatic α-keto acids. Edwards & Knox (379) have separated maleylacetoacetate isomerase from the hydrolase, and have demonstrated a glutathione requirement for the former; glutathione can catalyze the isomerization nonenzymatically but at a lower rate. The authors point out that this is the first enzyme described which catalyzes a *cis-trans* isomerization but, in view of its high specificity, they doubt whether it could catalyze similar isomerizations in vitamin A metabolism.

PROTEIN SYNTHESIS

Amino acid activation.—One locus for the energy requirement for protein synthesis (380) appears to be activation of amino acid carboxyl groups. Berg

(381), Hoagland and his co-workers (382, 383), and DeMoss & Novelli (384) observed this reaction as an amino-acid-dependent PP_i^{32} exchange into ATP. These studies have been extended; enzyme-bound amino acid-acyl adenylates appear to be intermediates. Studies in these and other laboratories have encompassed yeast, rat liver, pancreas, and a number of microorganisms; the results and interpretations have been remarkably uniform. The mechanism appears to be similar to that for activation of acetate (385), pantoate (386, 387), and butyrate. Thus it appears that formation of acyl adenylate represents an important pathway for carboxyl group activation.

Berg (381, 389) and Berg & Newton (390) have demonstrated a PP_i^{32}-ATP exchange reaction in yeast which is specifically dependent upon L-methionine. No exchange of AMP-C^{14} with ATP was observed and adenyl L-methionine was not isolated; however, with high concentrations of hydroxylamine, L-methionine hydroxamate formation was observed. The studies of Hoagland, Zamecnik and their co-workers (382, 383, 391) stem from their work on amino acid incorporation into protein of rat liver homogenates. These workers had demonstrated (392, 393, 394) that incorporation of labelled amino acids into rat liver microsomes *in vitro* was dependent upon ATP and enzymatic components of the soluble protein fraction. The function of this soluble fraction now appears to be carboxyl activation of amino acids (383). A preparation from rat liver contained enzymes which catalyzed PP_i^{32} exchange into ATP, dependent upon both the concentration and the number of amino acids added. Net formation of amino-acyl adenylate was not observed, but hydroxamic acids were formed in the presence of hydroxylamine, simultaneous with appearance of equimolar quantities of pyrophosphate. The authors postulate that adenyl-amino acids are firmly bound to the enzyme surface and net reaction can thus only be observed in the presence of a suitable acceptor, in this case hydroxylamine. Fractionation and heat inactivation studies as well as increased PP_i^{32} exchange in the presence of increasing numbers of amino acid species, suggest that different enzymes are responsible for activation of different amino acids. Similar exchange reactions have been observed by DeMoss & Novelli (384) in preparations from a variety of microorganisms. The enzymes were active toward the same eight amino acids in *S. hemolyticus* and *Neurospora* as well as in various strains of *E. coli*. Again, high concentrations of hydroxylamine caused the formation of corresponding amino acid hydroxamates. An increase in the total number of amino acids markedly increased PP_i^{32} exchange. Why these systems, in a number of microorganisms, may be specific for the same eight amino acids is not immediately apparent. DeMoss & Novelli suggest that the observed reaction represents a primary activating mechanism; amino acids which do not participate may be secondarily activated through some transfer reaction with one of the active amino acids.

Other studies of DeMoss & Novelli (395, 396, 397) have elucidated the mechanism of primary amino acid activation. Although net formation of amino acid adenylate was not observed, synthetic leucyl adenylate yielded

labelled ATP in the presence of PP_i^{32}; the reaction proceeded at least 50 per cent in the direction of ATP synthesis. Recently Davie, Koninsberger & Lipmann (398) have described PP_i^{32} exchange dependent upon a variety of amino acids in supernatants of beef pancreas; a specific tryptophan-activating enzyme, 70 to 80 per cent pure, was isolated. This protein contained bound GMP and tryptophan; again, net formation of adenyl-amino acid was not observed, but hydroxylamine in high concentration resulted in formation of amino acid hydroxamates. Improved methods for quantitative determination of amino acid hydroxamates, as described by Schweet (399), may facilitate further studies in this field.

The evidence to date indicates that amino acid activation may be formulated as follows:

(*a*) E + ATP + amino acid $\rightleftharpoons$ E-AMP-amino acid + PP_i
(*b*) E-AMP-amino acid + acceptor $\rightarrow$ E + amino acyl-acceptor + AMP.

In the studies cited, the "acceptor" has been hydroxylamine. Whether the natural acceptor is on the pathway of protein synthesis must await further studies; present evidence suggests that this may indeed be so. Hoagland *et al.* (383) have calculated that the rate of leucine hydroxamate formation in rat liver supernatants can account for the rate of incorporation of leucine into liver proteins *in vivo;* the widespread occurrence of this reaction is again suggestive of the importance of this mechanism. Should this mode of activation of amino acids prove to be essential in protein synthesis, it would bear out Lipmann's prediction (400) that phosphate bond energy of ATP may be used for carboxyl activation of amino acids in protein synthesis.

Other observations may bear upon the existence of activated amino acids. It cannot presently be stated whether the lag period for the incorporation of methionine-S^{35} into liver slices, observed by Ziegler & Melchior (401), and the abolition of this lag by aerobic preincubation, represents energy demands for amino acid transport into cells, protease action to yield suitable "substrate" for incorporation, or the time necessary to activate amino acids. The complex relationships observed by Eiduson & Dunn (402) on the inhibition of utilization of various phenylalanine derivatives by various phenylalanine analogues were interpreted by the authors as suggesting "activated" derivatives of aromatic amino acid metabolites.

Incorporation of amino acids into cellular subunits.—The amino acid incorporation system studied by Hoagland, Zamecnik and their co-workers (382, 391 to 394, 403, 404) consists of microsomes, proteins precipitable at pH 5 from rat liver supernatant, ATP, an ATP generator, and GDP or GTP. The pH 5 enzymes (383) are concerned with amino acid activation, and the ATP requirement is specific for this step; in the presence of microsomes and GDP or GTP, amino acids are incorporated into microsomal nucleoprotein, apparently in peptide linkage (391). Unlike PP_i exchange, amino acid incorporation is not accelerated by a mixed amino acid pool. Whether this indicates that incorporation of a single amino acid is truly in-

dependent of the presence of other amino acids, or whether microsomes may furnish small but adequate amounts of free amino acids has not been ascertained. GDP or GTP is required for incorporation; the preparation contains nucleoside diphosphokinase, and it cannot be stated which nucleotide is required. The authors suggest two possible roles for guanosine nucleotides: (*a*) transferring activated amino acids from enzyme-bound adenylate complex to RNA-protein in microsomes or (*b*) utilization of GDP-GTP to form a complex higher derivative which is itself a cofactor for the incorporation mechanism. Since the GDP-GTP effect is independent of the amino acid incorporated, this nucleotide effect appears to differ from the differential stimulation of incorporation of specific amino acids by various nucleotides, as observed by Gale (405).

Littlefield & Keller (404) have observed a similar incorporation system in cytoplasmic protein particles of Ehrlich ascites cells. In the presence of pH 5 enzymes from either liver or ascites cells, ATP, and GTP, these particles incorporated amino acids. An ATP-generating system was not required; the authors suggest that this reflects lower adenosinetriphosphatase activity than in liver microsomes. Webster & Johnson (406, 407) have reported amino acid incorporation by a particulate preparation from pea seedlings. The effect of nucleotides upon this system will be discussed later. Sachs & Neidle (408) have studied a rat liver microsomal incorporation system prepared from lyophilized microsomes and a supernatant fraction which appears to be equivalent to the amino acid activating system previously described. The latter authors, and Sachs & Waelsch (409) found that this system required an ATP generator and was stimulated by neutral salts and potassium; it was inhibited by high concentrations of ATP, AMP, and polyvalent anions. Pyrophosphate inhibition of amino acid incorporation was observed, accompanied by release of 10 to 15 per cent of the protein and 50 to 60 per cent of the RNA of microsomes. Addition of pyrophosphate to prelabelled microsomes similarly led to release of nucleotide-protein. This finding may have bearing upon the nature of amino acid linkage to the RNA of microsomes.

The question of whether this microsomal incorporating system reflects the process *in vivo* has been studied by Littlefield *et al.* (403, 404). These workers administered labelled amino acids *in vivo* and isolated a particulate RNA-protein fraction from microsomes of liver and Ehrlich ascites cells which contained RNA and protein in approximately equal proportions. In liver, this fraction was labelled earliest after amino acid administration *in vivo*, and it lost activity rapidly while other microsomal proteins slowly acquired radioactivity. Similar particles from Ehrlich ascites cells (404), showing two electrophoretic components, contained virtually all the amino acids which had been incorporated *in vivo*, and were able to incorporate amino acids *in vitro* in the presence of liver-activating enzymes. Thus, correlation between localization of incorporation *in vivo*, and incorporating activity *in vitro*, appears to be excellent.

The nature and function of the RNA-protein complexes described above is of paramount importance to future understanding of the mechanism of protein synthesis, since these appear to represent the earliest observable conjugate of amino acids in the incorporation process. In addition to the 1:1 RNA-protein microsomal particles isolated by Littlefield *et al.* (403), particles of similar composition have been observed in bacteria by Schachman, Pardee & Stanier (410). A ribonucleoprotein from pea seedlings, studied by Ts'o, Bonner & Vinograd (411), also has an RNA-protein ratio of 1:1, and may be a component of the pea seedling amino acid incorporation system studied by Webster & Johnson (406, 407). These "particles" had an apparent molecular weight of 4.5×10^6, and were rapidly aggregated by low concentrations of Mg^{++}; ATP (2×10^{-3} *M*) not only caused resolution of these aggregates but degraded the particles to an inhomogeneous mixture of lower molecular weight. These observations may be pertinent to the degradation of microsomal RNA-protein by pyrophosphate observed by Sachs & Waelsch (409). Potter & Dounce (412, 413) have studied nucleotide-amino acid complexes in alkaline digests of liver, pancreas, and yeast RNA. These complexes were alkali-stable and ninhydrin negative until heated with acid. The ratio of nucleotide to amino acid appeared to be close to unity; the authors propose a phosphoamide linkage between nucleotide and amino acid to explain the properties of this complex, and suggest that the bound amino acids may represent intermediate stages in peptide chain synthesis.

Additional, less direct, evidence has also accumulated that microsomes are an important site of protein synthesis. Akazawa (414) found that most of the increase of RNA and protein which accompanies infection of sweet potato slices occurred in the microsome fraction and Reid *et al.* (415, 416) found higher parallelism between pyrimidine and amino acid incorporation *in vivo* in microsomes than in other liver fractions of rats under various hormonal influences.

However, there is also evidence that microsomes may not be unique as a site for protein synthesis. Simpson *et al.* (417, 418) found slightly higher incorporation of amino acids into the mitochondria than into microsomes of muscle shortly after leucine-C^{14} injection; in liver, early incorporation was chiefly into the microsome fraction. Incorporation by muscle mitochondria *in vitro* was observed. Stephenson & Zamecnik (419) found that tobacco leaf-disc microsomes were inactive in incorporating amino acids, while a 15,000 g pellet, a 105,000 g supernatant, and chloroplasts were active. Allfrey, Mirsky & Osawa (420) observed chloramphenicol-insensitive amino acid incorporation by isolated calf thymus nuclei; the highest activity was found in a nonhistone protein associated with DNA. Whether such nonmicrosomal incorporation systems represent different locations for the same mechanism, or whether the mechanisms are different from those in microsomes, is as yet unknown.

Nisman *et al.* (421) have studied an apparently reversible incorporation of valine and methionine into protoplasts of *M. lysodeikticus*. This incorpo-

ration required adenine nucleotides, and was inhibited by addition of an amino acid mixture, by chloramphenicol, and by later incubation with C^{12} amino acids of the same species. The anomalous results previously obtained by Borsook *et al.* (422, 423) on incorporation of lysine by guinea pig liver preparations have been partially explained by Schweet (424), who demonstrated that lysine is incorporated at the ϵ- rather than the α-amino group; the mechanism remains obscure. The impression that the fructose-amino acid stimulation of protein synthesis in rabbit reticulocytes observed by Borsook *et al.* (425) is due to facilitation of iron transport is strengthened by the observation of Neiland & Townsley (426) that fructose-phenylalanine and versene were both effective in supporting growth of a strain of *M. lysodeikticus* which requires iron-binding substances. Whether fructose-amino acids are normal metabolites, or artifacts similar to amino acid-sugar complexes formed on heating, remains obscure.

In what may be a highly significant communication Straub, Ullmann & Acs (427) report protein synthesis by a solubilized system. These workers had previously observed amylase synthesis in a granular fraction of pigeon pancreas in the presence of amino acids and a high concentration of ATP. The present system was derived from a water extract of pigeon pancreas acetone powder; in the presence of .015 *M* ATP, ascorbate, and casein hydrolysate, amylase synthesis was observed. This was inhibited by *p*-fluorophenylalanine and ribonuclease. Should this preliminary study be confirmed, it may render the problem of protein synthesis accessible to the classical tools of enzymology.

Nucleic acid and protein synthesis.—The concept that RNA is intimately related to protein synthesis has evolved from a long series of cytochemical and biochemical observations, and is supported by a vast body of indirect as well as direct evidence (428). The amino acid incorporation observed in microsomal RNA-protein (404, 406, 407), and the observation that the RNA-protein of microsomes appears to be the earliest labelled material after administration of radioactive amino acids *in vivo* (403) is itself strong evidence that the initial steps in protein synthesis occur in close conjunction to a ribonucleic acid site.

Ribonuclease has continued to be widely used in studying RNA-protein synthesis relationships. This enzyme was found to prevent enzyme induction (429) and protein synthesis (430) by protoplasts of *B. megatherium.* In the latter studies, removal of ribonuclease allowed apparent recovery of glycine incorporation, after resynthesis of RNA. Ribonuclease inhibition of amino acid incorporation into protoplasts of *M. lysodeikticus* has been observed by Bridoux & Hanotier (431), and by Nisman *et al.* (421). The previously cited synthesis of amylase (427) is inhibited by ribonuclease. However, Ledoux (432) has described complex effects of ribonuclease on Landshutz ascites tumor cells at various stages of age and growth; under some circumstances an actual increase in intracellular RNA content accompanied ribonuclease action. This finding suggests that when ribonuclease is used as an

experimental tool, the assumption that it removes RNA may not always be warranted.

Additional evidence for an RNA-protein relationship in synthesis is furnished by successful attempts to induce enzyme formation by addition of RNA of preinduced organisms to unadapted cells in absence of specific inducer. Kramer & Straub (433) found that addition of RNA, from a penicillinase-containing strain of *B. cereus*, to cells of a penicillinase-lacking strain induced enzyme formation in absence of penicillin. Enzyme formation was not induced when the RNA was pretreated with ribonuclease, when ribonuclease was present during the induction period, or when the RNA originated from a penicillinase-lacking strain. However, pretreatment of unadapted cells with ribonuclease shortened the induction period or increased the amount of enzyme induced, particularly when specific RNA rather than penicillin was the inducer. The authors interpret the effect of ribonuclease pretreatment as making precursors available for subsequent resynthesis of specific RNA. Induction of enzyme formation by RNA was more rapid than that by penicillin, but it soon ceased, presumably because of degradation of specific RNA, which cannot be synthesized in the absence of penicillin. The observation that ribonuclease pretreatment of bacterial RNA abolishes its effectiveness as an enzyme inducer contrasts with the findings of Reiner & Goodman (434) who found equal activity of RNA and ribonuclease digestion products from gluconate-adapted *E. coli* in inducing enzyme in lactate-grown cells. The reason for this discrepancy is not immediately apparent. Hunter & Butler (435) found that addition of RNA from lactose-induced *B. megatherium* was able to induce β-galactosidase formation in uninduced bacteria. The authors state that results were often erratic and a small amount of induction was occasionally obtained by addition of RNA from unadapted organisms. The purine antagonist azaguanine inhibits induced synthesis of β-galactosidase by *S. aureus* (436); this inhibition is overcome by guanine.

Webster & Johnson (406, 407) have conducted studies of amino acid incorporation into pea-root particles, similar to those which Gale conducted on disrupted bacteria (405, 437, 438); incorporation of glutamate was inhibited by pretreatment of the enzyme with ribonuclease and this inhibition was reversed by pea-seed RNA. Deoxyribonuclease treatment was ineffective, as was DNA addition to untreated particles. Breakdown products of RNA, purines, and pyrimidines (including thymine) stimulated glutamate incorporation; maximum stimulation was obtained by a mixture of ATP, GTP, CTP, and UTP (407). These nucleotides permitted measurable increase in the protein and RNA of the particles in the presence of 17 amino acids, Mg^{++}, and K^{+}; ATP^{32} was simultaneously incorporated into nucleic acid. Major discrepancies between these studies and those of Gale (405, 437, 438) appear to lie in the ineffectiveness of DNA or deoxyribonuclease with pea-root particles (although the effectiveness of thymine remains unexplained). Gale observed that specific nucleotides stimulated the incorporation of specific amino acids, while Webster & Johnson (406, 407) found equal

stimulation of incorporation of all amino acids tested, although this stimulation varied with the nucleotide added. Gale (437) had found that glutamic acid incorporation was dependent upon endogenous RNA and DNA; utilization of smaller fragments for β-galactosidase synthesis appeared to vary with the state or resolution of his system. At intermediate stages of resolution purines and pyrimidines appeared to suffice, but at highest resolution DNA was uniquely effective, although ribonuclease inhibited DNA stimulation. The chief obstacle in evaluating these studies is the uncertainty concerning the quantitative effect of "resolution" procedures upon residual RNA and enzymatic components of the sytems studied. The question of whether effectiveness of "foreign" nucleic acids and nucleic acid degradation products represent direct effects of these products, or provision of materials for new nucleic acid synthesis, perhaps dependent upon the presence of small amounts of endogenous DNA ("template") is still not clearly answered; Webster & Johnson (406) did not report the effects of "foreign" RNA or nucleotides upon ribonuclease-treated pea-root particles.

Webster (407) observed that glutamic acid incorporation into pea-seed particles was inhibited by nucleic acid antagonists, and conversely, amino acid analogues inhibited RNA synthesis, but Chantrenne (439) observed that *p*-fluorophenylalanine, which prevented the production of cytochrome and catalase (which occurs when anaerobic yeast is exposed to oxygen), failed to inhibit incorporation of adenine and uracil into RNA. Since amino acid analogues (*vide infra*) may act by causing synthesis of inactive protein rather than by blocking protein synthesis, this observation does not necessarily negate association of RNA synthesis and protein synthesis.

Studies of correlation between growth and RNA synthesis continue. Mandel (440) observed incorporation of radioactive azaguanine by *B. cereus* in the presence of casein hydrolyzate; little was incorporated when cells were grown on glucose-salts medium, but addition of aspartate, which increased growth, also increased azaguanine incorporation. Laird & Barton (441) have measured the RNA content of normal and malignant tissue of rats. Tissues of high protein-synthesizing activity had a high RNA content in microsomes; tumor cells had highest RNA in the nuclear fraction, suggesting that malignant cells may have extra-microsomal sites for protein synthesis. Løvtrup (442) found that the ratio, protein synthesis/nucleic acid synthesis, was higher in *E. coli* adapting to lactose than in growing cells, and interpreted these results as indicating that synthetic activity during adaptation is quantitatively different from that during normal growth. Ben-Ishai & Volcani (443) have continued the studies from Spiegleman's laboratory (444, 445) which attempt to answer the question of whether cosynthesis, or merely presence of RNA, is required for protein synthesis. There is a ratio of three or four to one between the amount of cytoplasmic protein and RNA synthesized by a thymineless mutant of *E. coli* without DNA cosynthesis. Partial inhibition of RNA synthesis by purine and pyrimidine

analogues was accompanied by proportional inhibition of protein formation, while chloramphenicol inhibition of protein formation did not affect RNA synthesis. From these data, the authors conclude that protein synthesis is dependent upon RNA cosynthesis. Whether these studies reflect dependence of protein synthesis upon RNA synthesis, or merely suggest that the composition of the cell remains constant, by means of undefined feed-back mechanisms, cannot be determined. It is not impossible that inhibition of growth by purine antagonists may be found to yield proportional inhibition of apparently extraneous processes such as lipide synthesis, water transport, etc.

Chloramphenicol promises to be a powerful tool in studying relationships between RNA and protein synthesis. This drug inhibits protein synthesis without inhibiting nucleic acid synthesis, as demonstrated in *S. aureus* (446), in *E. coli* (447), in a thymineless *E. coli* mutant (443), and in bacterial protoplasts (421). *p*-Fluorophenylalanine uptake by *E. coli* is also inhibited by this drug (448). Hopps *et al.* (449) have shown that this inhibition of protein synthesis is not due to the action of chloramphenicol as an amino acid antagonist, nor is its action attributable to inhibition of amino acid activation (397), or intracellular amino acid concentrating mechanisms (450). Thus, chloramphenicol appears to "uncouple" protein synthesis from RNA synthesis. Recent studies by Pardee & Prestidge (451) and Gros & Gros (452) have demonstrated that RNA synthesis in the presence of chloramphenicol requires the presence of amino acids, even in the absence of protein synthesis. A number of amino acid-less *coli* strains were used in both studies; in the total absence of required amino acids, protein and RNA synthesis were proportionately limited, and chloramphenicol was without effect. In the presence of traces of amino acid, proportionality between RNA and protein synthesis was abolished by chloramphenicol, with marked stimulation of RNA synthesis and inhibition of protein synthesis. Since each of these mutants may be presumed to have all amino acids except the one for which it is blocked, these observations indicate that the complete amino acid spectrum is required for RNA synthesis just as for protein synthesis, even though protein synthesis does not actually occur. Gros & Gros (452) suggest that the amino acid requirement may be for specific transport of nucleotides to other amino acids or to other mono- or polynucleotides. Pardee & Prestidge (451) prefer to think of these observations as evidence for an RNA-amino acid "template" operative for both RNA and protein synthesis. These authors suggest that amino acids and nucleotides assemble on DNA or RNA nucleoprotein "template" surfaces, and the binding of bases in proper sequence would depend on the presence of mutually complementary amino acid, and vice versa. This process, presumably, would be insensitive to chloramphenicol which would interfere specifically with bond formation between the amino acids; the RNA-amino acid "template" would still be functional for RNA synthesis.

Thus, a "template" is implicit in the above observations, and chlor-

amphenicol appears to inhibit protein synthesis while permitting "template" accumulation to continue; a lack of amino acids inhibits both processes. Whether the physical counterpart of this hypothetical "template" is represented by the RNA-protein complexes which incorporate amino acids in microsomal preparations remains to be established. It would be of interest to know whether this incorporation is inhibited by chloramphenicol.

A different type of "dissociation" of amino acid incorporation into apparently separate components has been observed by Rabinovitz, Olson & Greenberg (453, 454) in studying the effect of methionine sulfoxide on incorporation of amino acids into proteins of Ehrlich ascites cells. A portion of the amino acid incorporation appeared to require all amino acids, including glutamine; since these cells degraded glutamine faster than they could synthesize it, addition of this amino acid stimulated incorporation of other amino acids, and methionine sulfoxide, which inhibits glutamine synthesis, inhibited this incorporation. Inhibition was reversed by glutamine in a noncompetitive manner. Incorporation of all amino acids tested was affected similarly by methionine sulfoxide and glutamine, but about 50 per cent of the incorporation was insensitive to this inhibitor even at high concentrations. Thus, the methionine sulfoxide-sensitive portion of amino acid incorporation required all amino acids, while the methionine sulfoxide-insensitive portion did not. The authors suggest that the uninhibitable fraction may represent some process other than protein synthesis. It would be of interest to correlate methionine sulfoxide-insensitive incorporation with the postulated "template" synthesis permitted by chloramphenicol.

This picture of an RNA-amino acid "template" has obvious limitations. It includes no role for DNA, nor does it predict enzymatic requirements for protein synthesis. Whether the hypothetical "template" is closely associated with protein-synthesizing enzymes, or whether an RNA-amino acid "template" has inherent catalytic properties is unknown. Although Binkley *et al.* (455) have claimed peptidase activity for nucleic acid-amino acid complexes, these observations require further documentation and confirmation.

Several observations do not directly confirm the concept of a close relationship between RNA and protein synthesis. Hokin & Hokin have found that stimulation of pancreatic amylase synthesis by amino acids was not accompanied by an increase in RNA content (456) or by an increased P^{32} incorporation into RNA (457), nor has the stimulation of amylase synthesis in mouse pancreas by preirradiation of mice been accompanied by changes of RNA concentration or P^{32} incorporation *in vitro* (458); Sidransky & Farber (459) have found that administration of ethionine increased subsequent capacity of rat pancreas to synthesize amylase without change in pancreatic nucleic acid concentration. However, Younathan & Frieden (460) have observed that amylase synthesis by pigeon pancreas slices *in vitro* was unaffected by chloramphenicol; if pancreas cells are permeable to this drug, this may suggest that protein synthesis in pancreas may occur by mechanisms different from those of other systems studied.

Another chloramphenicol-insensitive amino acid incorporation system has been described by Allfrey, Mirsky & Osawa (420) in isolated calf thymus cell nuclei. This system appeared to be dependent upon DNA; protein synthesis was inhibited by deoxyribonuclease, and the amount of inhibition appeared to be proportional to the amount of nucleic acid removed. When the protein of nuclei which had been exposed to radioactive amino acid was fractionated, the highest activity was found in a nonhistone protein associated with DNA. The DNA-dependency contrasts with the findings of Webster & Johnson (406, 407) in pea seedlings and suggests fundamental differences. The usual concept of protein synthesis does not implicate DNA directly; additional evidence for nondependence of protein synthesis upon DNA is furnished by Wainwright & Nevill (461) who observed that thymine deficiency in a thymineless *E. coli* mutant did not inhibit induction of nitrate reductase, although amino acid deficiency in amino acid-less mutants inhibited induction.

The nature of the building blocks for protein synthesis.—The question of whether protein synthesis occurs by "simultaneous" condensation of amino acids, or whether this process occurs by stepwise synthesis of protein fragments, either free or in conjugation with a "template," has not received a categorical answer. The problem has been attacked by determining whether various residues of the same amino acid within a protein are incorporated simultaneously from a free amino acid pool (constant specific activity in various portions of the protein molecule, or constant ratios of specific activity of two different amino acids in two different proteins), or whether the amino acid precursors for the various positions of the same amino acid in protein are diluted to varying extents with intermediates. Observations of unequal labelling along a peptide chain, however, are only incompatible with the "simultaneous" hypothesis if the completed protein molecule cannot return to an "exchange" site to exchange an amino residue at a later time when the activity of that amino acid in the precursor pool has changed. Thus, an understanding of whether the later process can indeed occur is fundamental to the interpretation of such studies.

These problems have been discussed by Steinberg, Vaughan & Anfinsen (462) who propose a mechanism for protein synthesis which involves stepwise condensation of amino acids to form "template"-bound intermediates. Additional studies have appeared during the past year and, as previously, their conclusions have been contradictory. Studies by Velick (463) and Godin & Work (464) are compatible with "simultaneous" condensation. Velick (463) has extended the previous studies of his group (465, 466) on amino acid incorporation into purified muscle proteins by studying specific activity ratios of phenylalanine/tyrosine in rabbit muscle aldolase, triose phosphate dehydrogenase, myosins, and actin, as well as serum albumin and fibrinogen. Specific activity ratios for the enzymes, actin, and H-meromyosin were essentially equal, suggesting origin from the same amino acid pool. Myosin grossly appeared to have been made from a different pool, but the author

suggests that this represents different turnover rates of H- and L-meromyosins and their different phenylalanine and tyrosine contents. Serum albumin and fibrinogen appear to come from different pools than muscle protein; the author suggests that this difference reflects hepatic synthesis of both tyrosine and serum proteins; the liver pool may be expected to have a higher specific activity ratio for tyrosine/phenylalanine than extrahepatic pools when labelled phenylalanine is administered. However, this does not appear to explain the observed difference in the activity ratios of serum albumin and fibrinogen from each other. Godin & Work (464) have extended their previous studies with Askonas *et al.* (467) on synthesis of milk proteins by the goat, where equal specific activities of an amino acid from various parts of the protein chain had been observed. The present studies noted that various lysine peptides derived from goat casein failed to alter the equal specific activity of lysine residues in various portions of the protein chain, suggesting that circulating peptides cannot be used for casein synthesis without prior degradation. However, only 65 mg. of peptide-lysine were administered, and it is not known what proportion was available as peptide to the mammary gland. Therefore, these studies cannot be taken as definitive evidence for nonparticipation of peptides.

Although the studies just cited are compatible with "simultaneous" synthesis, a number of studies have appeared which are more readily interpretable in terms of a "sequential" mechanism. Gehrmann, Lauenstein & Altman (468) report more rapid degradation of C-terminal than nonterminal glycine in collagen of rats prefed glycine-C^{14} over an eight-day period. Shimura (469) claims that the N-terminal glycine of silk fibroin was nine to ten times as radioactive as nonterminal glycine following injection of C^{14}-glycine into the body fluid of the silkworm *Bombyx mori*. However, interpretation of these results is completely clouded by an obvious printing error. The reviewers' best guess as to the meaning of a legend under a table is that the authors did not correct for self-absorption. Were this so, the amount of sample available for counting being greater for nonterminal than N-terminal glycine, the lower specific activity of the former may have reflected greater self-absorption rather than lower activity. This question can only be decided when a fuller account of this work appears. Rychlik & Sôrm (470) found that protease synthesis by mouse pancreas slices was stimulated more by a peptic hydrolyzate of chymotrypsin than by a tryptophan-fortified total acid hydrolyzate, partial acid hydrolyzate, or amino acid mixture. They suggest that chymotrypsin-derived peptides are involved in pancreatic protease synthesis. Grodski & Tarver (471) and Light & Simpson (472) have developed paper chromatographic systems to isolate insulin from small amounts of pancreas. This may permit labelling studies with a protein of known amino acid sequence, and thus facilitate further progress in this field.

Steinberg *et al.* (462) have criticized the studies of Velick and his group (465, 466) and those of Askonas *et al.* (467) for not having obtained sufficient-

ly early samples, since according to their kinetic analysis (462) uniform specific activity is obtainable even with sequential synthesis as the time period from administration of label to isolation of protein is prolonged. Observations of nonuniform labelling are considered more critical; whereas equal labelling under some circumstances is compatible with "sequential" synthesis, unequal labelling in no circumstances is compatible with a hypothesis of "simultaneous" condensation. However, this interpretation is valid only if a completed protein cannot, after its synthesis, subsequently exchange an amino acid.

Protein "turnover."—The concept of "turnover" arose from the experiments of Schoenheimer (473) which indicated that ingested amino acids appeared in constitutive body fractions which had hitherto been assumed to be stable. The findings of Schoenheimer were macroscopic; protein "turnover" was observed in the whole animal, or in whole organs, and analyses were conducted on heterogeneous fractions. As these studies have been extended to defined molecular species, it has become apparent that there is a wide diversity in rate and extent of "turnover" among various proteins. Further, the observation of incorporation of a component into a larger molecule does not define the mechanism involved; exchange reactions at an enzyme surface, e.g., "turnover" of ATP-pyrophosphate, and "turnover" of hemoglobin by net synthesis and irreversible red cell destruction, represent extremes of the types of mechanisms which could account for the incorporation of a labelled amino acid into a protein. Thus, the question of protein turnover cannot be divorced from the mechanism of protein synthesis. Tarver (474) has reviewed the extensive data available on protein "turnover," and has examined and discussed possible mechanisms.

The problem of utilization of "old" protein for new protein synthesis has been brought into sharp focus by the studies of Spiegelman *et al.* (445, 475) and Hogness, Cohen & Monod (476, 477). These groups found that adaptive enzymes in *E. coli* were formed almost exclusively from amino acids in the medium, with virtually no contribution from preformed cellular protein. This observation led Hogness *et al.* (476) to suggest that an observed dynamic steady state may result from irreversible degradation of protein with equal resynthesis from exogenous precursors and, in the whole animal, observed "turnover" may represent irreversible destruction of an individual cell and synthesis of a new cell from new exogenous components. Regardless of the validity of this suggestion, the above studies with adaptive enzymes have forced a reappraisal of the meaning of the term "dynamic steady state."

The findings from the laboratories of Spiegelman and Monod have stimulated a number of other studies in bacterial and mammalian systems in an attempt to determine the generality of the former observations. Koch & Levy (478) have prepared *E. coli* with labelled protein-glycine and unlabelled purine by growing these cells in the presence of glycine-C^{14} and unlabelled purine; the presence of the latter inhibits purine synthesis *de novo*.

Subsequent washing of the cells, followed by incubation in a purineless glucose medium indicated that virtually no protein glycine was used for purine synthesis, indicating almost complete absence of protein breakdown during growth. Markowitz & Klein (479) have studied the induced synthesis of amylase and sucrose phosphorylase in *P. saccharophilia*. Previous induction of one enzyme inhibited subsequent induction of the other; this inhibition was overcome by casein hydrolyzate, suggesting that once the internal free amino acid pool was depleted, external amino acids were required for further enzyme synthesis. However, experiments with cells prelabelled with glucose-C^{14} indicated higher utilization of cellular protein for induced enzyme synthesis than was apparent in the studies of Spiegelman and of Monod. In growing cells, about 16 per cent of new enzyme was formed from preformed cellular constituents; in resting cells, 40 to 65 per cent. It is noteworthy that this organism, unlike *E. coli*, contains an intracellular amino acid pool. The observation of Cowie & Walton (177) that cells of *T. utilis*, whose amino acid pool was labelled by brief exposure to fructose-C^{14}, lost no radioactivity while this pool was converted to protein over a four-generation growth period, indicates that cell protein could not have been degraded and used as an energy source.

Loftfield & Harris (480) studied the source of amino acids for ferritin synthesis in the liver of rats. After iron oxide administration, labelled valine, isoleucine, and leucine were infused continously for periods up to three days; liver ferritin was then isolated and degraded. The specific activities of free intracellular valine, isoleucine, and leucine were found to be identical with those of the corresponding amino acids in newly-formed ferritin, but not identical with those in whole liver protein. Although the data do not permit evaluation of the contribution of liver protein to newly synthesized ferritin they emphasize the importance of free amino acids. The authors suggest that their evidence complements that of Askonas *et al.* (467) and of Velick *et al.* (465, 466) in indicating protein synthesis from free amino acids. However, these studies may also be subject to the criticism which Steinberg & Anfinson (462) cited for the studies of the aforementioned groups: the time interval used by Loftfield & Harris might have permitted equilibration of the free amino acid pool with some intermediate peptide or labile protein which itself was a ferritin precursor.

Junqueira, Hirsch & Rothschild (481) injected glycine-C^{14} into rats and compared the radioactivity in plasma protein and pancreatic juice proteins. The finding that the peak radioactivity of pancreatic juice was 10 to 20 times higher than the peak radioactivity of plasma proteins is consistent with free amino acids rather than serum protein being the precursor of pancreatic juice proteins; the only alternative explanation would be the presence of small quantities of a highly radioactive plasma protein.

Although the series of studies cited above are consistent with the hypothesis (445, 475, 476, 477) that cellular proteins are not used for new

protein synthesis, this hypothesis is by no means accepted by all workers in this field. Thus, Moldave (482) suggests that the absence of "turnover" observed by Spiegelman and by Monod in *E. coli* should not be considered as true for all cells. He has observed that prelabelled Ehrlich ascites cells, implanted intraperitoneally in a dialysis membrane in mice, lose radioactivity to the medium even though their total protein content remains the same. Under the conditions of this incubation, cells divide but do not grow; whether this "turnover" is a function of this type of physiological behavior cannot be determined.

Similarly, the conclusion of Junqueira *et al.* (481) that plasma proteins are not the source of pancreatic juice proteins is at variance with other studies, which suggest direct utilization of plasma proteins for protein synthesis. Thus, Babson & Winnick (483) found that carrier amino acids failed to dilute out the corresponding radioactive amino acid in plasma protein in the course of tissue protein synthesis, while Yuile *et al.* (484) observed that plasma protein lost little lysine-C^{14} as CO_2 during transfer of the label to tissue proteins. Studies such as those of Babson & Winnick (483) which depend upon dilution of a protein amino acid by a carrier amino acid have come under serious criticism by Loftfield & Harris (480), who found that intracellular free amino acids did not exchange freely with administered amino acids; the amino acid pool for protein synthesis, therefore, may not be accessible to carrier amino acids.

Thus, whether plasma proteins can be "rearranged" for protein synthesis without prior degradation to free amino acids remains a subject of controversy. The situation is aggravated by the possibility that these proteins, rather than being "typical," may have a unique role, so that results obtained with plasma proteins may not be safely extrapolated to other proteins. The known high rate of plasma protein synthesis, and the nutritional availability of these proteins when administered intravenously, suggest a special function. This suggestion is strengthened by the surprising observations of Roberts & Kelley (485) that phenylalanine-labelled rat serum albumin was a substrate for oxidation by rat liver slices and the specific activity of CO_2 formed by slices respiring in labelled albumin equalled that of the serum protein in the medium. Denatured albumin, or human or bovine albumin, did not maintain respiration of slices in a manner similar to native rat albumin.

"Active" degradation of protein.—A dynamic steady state requires that the rate of degradation of protein must equal the rate of synthesis, and an understanding of the former process is also necessary for a clear picture of "turnover." Several studies within the past year have indicated that intracellular protein degradation may not proceed by simple catheptic hydrolysis. The original studies of Simpson (486) indicated that cyanide, DNP, and anaerobiosis inhibited the release of radioactive amino acids from liver slices of rats who had received methionine-C^{14} or leucine-C^{14} *in vivo*. These studies

have been confirmed and extended by Steinberg & Vaughan (487, 488), who measured release of radioactive amino acids and total NPN from liver and kidney slices of rats which were fed C^{14}-labelled phenylalanine, alanine, or lysine. Release of radioactive amino acids was inhibited not only by anaerobiosis and dinitrophenol, but by *o*- and *p*- fluorophenylalanine. Phenylalanine antimetabolites inhibited the release of phenylalanine, alanine, and lysine to the same extent; autolytic degradation of liver slice protein at pH 5 was unaffected by these agents. Similarly, Schweiger & Rapoport (489) noted a dinitrophenol inhibition of release of NPN by incubated rabbit reticulocytes. Steinberg & Vaughan (488) consider that the demonstration of an energy demand for protein degradation is compatible with reversibility at one or more steps of protein synthesis, as is inhibition of degradation by phenylalanine analogues. A fundamental obstacle to the interpretation of these studies is our ignorance of the immediate precursor of the radioactive amino acids which are released. Since the total nitrogen released represented about 4 per cent of total liver protein (487), the question arises whether this release was shared by all proteins or represented degradation of a specific component. The possibility remains that these amino acids were released, not from a constitutive protein of liver, but from an RNA-amino acid "template," and that the reversibility may occur at the site of "template" formation rather than protein synthesis, if the previously noted chloramphenicol studies indeed permit one to make this distinction.

The state of intracellular amino acids.—The relationship of the intracellular free amino acid pool to protein synthesis and to externally administered amino acids has come under critical scrutiny. The studies of Loftfield & Harris (480) indicate that, although this pool is available for protein synthesis, it is not freely exchangeable with externally administered amino acids. Even after three days of continuous infusion with sufficient C^{14}-labelled valine, leucine, or isoleucine to represent 10 to 20 times the content of that free amino acid in the entire rat (or up to double the content of the corresponding amino acids in total liver protein), the specific activity of "free" intracellular amino acid was only 10 to 20 per cent of that of the amino acid administered. This specific activity appeared to be a limiting value; although the data, using different rats for different time points, necessarily lack complete precision, it appears that "equilibration" was complete after 4 to 24 hr. of infusion. This finding, combined with the observation that newly synthesized ferritin had the same specific activity as the intracellular amino acids, is reminiscent of the Folin concept of separate "endogenous" and "exogenous" pathways: administered amino acids may become part of an "exogenous" pool for oxidation whereas only a relatively small portion may be incorporated into the "endogenous" pool for protein synthesis. Cowie & Walton (177) have also concluded that apparently "free" (cold TCA-soluble) amino acids in *T. utilis* seem to lie exclusively in a pathway toward protein synthesis. These authors exposed yeast briefly to

radioactive fructose, permitting labelling of intracellular "free" amino acids; the organisms were then washed and rate of incorporation of label into protein and nucleic acid was followed. Incubation of prelabelled organisms with unlabelled amino acids did not affect the rate of incorporation of labelled amino acids into cellular proteins. These data were interpreted as indicating the TCA-soluble amino acids are not "free" but are adsorbed to certain sites intimately concerned with protein synthesis. Similar conclusions were reached by Britten, Roberts & French (450) who observed rapid binding of proline-C^{14} into the TCA-soluble fraction of *E. coli*. This binding was unaffected by other amino acids or chloramphenicol, but was inhibited by pretreatment of cells with proline-C^{12}. Steward, Bidwell & Yemm (50), by growing carrot root cultures in the presence of radioactive glutamine and glucose and measuring specific activity of intracellular glutamine and glutamic acid, have obtained data consistent with the existence of a large stored pool of glutamine, and a small active pool which is in equilibrium with glutamic acid.

That some selection device must be interposed between the cellular amino acid pool and finished protein is apparent from the dissimilarity in composition between intracellular amino acids and tissue proteins in mammalian tissues (490), and in *T. utilis* (177) and from the dissimilarity between the amino acid composition of RNA-protein of silk gland and silk fibroin. However, Wu & Hogg (492) have observed similarity in amino acid composition of free and protein amino acids in *Tetrahymena pyriformis*.

Transpeptidation.—No evidence has accumulated during the year to suggest that transpeptidation plays a role in amino acid activation or protein synthesis. This subject has recently been reviewed by Adelberg & Rabinovitz (51) and Fruton (380). Barry (493), using doubly-labelled glutamine and C^{14}-glutamic acid, found that these amino acids were incorporated into casein independently, and that transpeptidation could not have been a significant mechanism in incorporation. Previously cited studies of Rabinovitz *et al.* (454) on the role of glutamine in protein synthesis by ascites tumor cells indicate that glutamine stimulation was due to the necessity for that amino acid as a protein constituent, rather than as a catalyst.

Rouser, Jelinek & Samuels (494) have observed transpeptidation between glutamine and glutathione in various types of human blood cells, and in canine leucocytes. Levin, Berger & Katachalski (495) have studied hydrolysis and transpeptidation of lysine peptides by trypsin. Ball, Revel & Cooper (496) have devised a manometric technique for the measurement of γ-glutamyl transpeptidase. Haurowitz & Horowitz (497) suggest that uptake of S^{35}-thiocyanate by protein may occur through formation of thiohydantoin rings with γ-glutamyl peptides, and suggest this technique as a means of detecting these peptides. Poly-γ-glutamic acid gave a much higher thiocyanate uptake than did the poly-α-acid.

Synthesis of "foreign" protein.—A wide variety of amino acid analogues

have been shown to inhibit protein synthesis and growth; although there is no need to postulate that all amino acid analogues inhibit by the same mechanism, recent studies have indicated that, in many cases, these analogues may act by being incorporated into an "abnormal" protein which may be biologically inactive. Gross & Tarver (498) found that ethionine-C^{14} was incorporated into growing cultures of *Tetrahymena*, and the label was found in combined form after partial acid hydrolysis. Pardee, Shore & Prestidge (499) found incorporation of azatryptophan and other tryptophan analogues into *E. coli* and T_2-phage. Growth of *E. coli* ceased after the number of initial cells doubled, and some enzymes normally present were found to be inactive. Munier & Cohen (448) observed *p*-fluorophenylalanine incorporation into *E. coli;* growth, valine-C^{14} uptake, and β-galactosidase induction were found to be linear rather than exponential; incorporation of analogue was inhibited by chloramphenicol. In the presence of thienylalanine and 4- or 5-methyl-tryptophan, similar growth effects were observed, but β-galactosidase was not formed. The authors concluded that analogues acted, not by blocking protein synthesis, but by causing synthesis of proteins abnormal in structure and function. These types of studies have been extended to mammals by the observations of Black & Kleiber (500) who found that norleucine-C^{14} administered to cows led to production of casein containing norleucine. Incubation of milk with labelled norleucine did not lead to uptake of label, nor was norleucine found in normal casein hydrolyzate. This incorporation of foreign amino acids into proteins finds its counterpart in the known ability in incorporate foreign purines into nucleic acids, and indicates that synthetic mechanisms, as well as degradative ones, can utilize "abnormal" substrates to produce "abnormal" material. It is of interest that Berg (389) observed that the yeast methionine-activating enzyme catalyzed slow pyrophospate exchange in the presence of ethionine, ATP, and PP_i^{32}, suggesting that this analogue may substitute for methionine in amino acid activating reactions.

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THE NUCLEIC ACIDS[1,2]

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Previous reviews of this topic have summarized progress in the biosynthesis (43), preparation (5), and organic chemistry of nucleic acids and nucleotides (37). This essay is concerned with recent work that deals with nucleic acid structure, relation to physiological and metabolic events, and biological interventions. The large increase in the number of published papers is met by an attempt to summarize rather than to expound in detail.

Structure and Chemistry

The ability to prepare DNA from various sources in a more purified and less degraded form and ever-increasing apparent molecular weight (100), coupled with an appreciation of the roles of hydrogen bonding and internal charge distribution, which underlie the structure proposed by Watson & Crick (305), have inspired re-examinations of the physicochemical characteristics of DNA with improved methodology.

Titration.—More accurate determination of the pG' values of the effective groups in herring sperm DNA by Cox *et al.* (71) indicates essentially no phosphoryl titration in the neutral range, which eliminates consideration of secondary phosphoryl end groups and thus the need to invoke triply esterified phosphate. The hysteresis (difference between forward and backward titrations) in the titration of both herring sperm (71) and thymus (48, 166) DNA is ascribed to the normal (A to T, C to G) hydrogen bonding, which is eliminated at the extremes of pH, in accordance with theory. The increase in ultraviolet absorption (hyperchromic effect) and bathochromic shift in the spectrum after DNA has been carried to pH 2 or 13 is also in accord (191). It has been indicated by Cavalieri & Rosenberg (46) that the acceptance of a proton by guanine, the apparent pK of which varies considerably with ionic strength (46, 166), may cause rearrangements in the purine that result in breaking both guanine-cytosine hydrogen bonds. The same reaction is held accountable by Cavalieri *et al.* for a spectrophotometrically

[1] The survey of the literature pertaining to this review was completed in November, 1956.

[2] The following standard abbreviations are employed: RNA and DNA for the ribo- and deoxyribonucleic acids; A, G, C, MC, U, and T for adenine, guanine, cytosine, methylcytosine, uracil, and thymine; AMP, ADP, etc., for the corresponding nucleoside 5′-phosphates and -pyrophosphates; and TMV for tobacco mosaic virus.

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observed irreversible denaturation of highly polymerized DNA by exposure to salt solutions below 10^{-3} *M* and less than pH 7 (47). Although the amino group of guanine had not earlier been implicated directly in interchain hydrogen bonding by either theory (305) or experiment (166), a reconsideration of bond angles and distances leads Pauling & Corey to the conclusion that it may be involved in a third hydrogen bond between guanine and cytosine (247). However, the precision with which the bond lengths are known leaves open the possibility that only two of these are involved in the internal twin-helix structure and that the third may be involved in external interactions. The large shifts in p*G'* values of guanine, cytosine, and adenine (48, 166) with change in concentration of nucleate or salt are interpreted (48) as evidence of further interaction between macromolecules.

Titrations of a bacterial RNA by Cox *et al.* (69) show a similar hysteresis, indicating that as much as 30 per cent of the 6-amino and 6-keto groups of the preparation are hydrogen bonded. In agreement with the results on DNA, no appreciable secondary phosphoryl groups are demonstrable (69).

The conclusions that the phosphates in the double helix model are wholly primary and occupy exposed positions are borne out by studies on the binding of acriflavine (150) and rosaniline (190). From the former study, it appears that the binding sites are heterogeneous; the curves can be described in terms of a two-constant equation. The open structure of the twin-helix model is shown by its permeability to water and sucrose molecules (169) and to the dye molecules, the hydrocarbon portion of which can slip between the parallel planes of the bases with strong van der Waals binding (150).

The degradation of DNA.—The macromolecular changes in thymus DNA during the course of its degradation by acid, heat, and deoxyribonuclease were earlier summarized by Dekker & Schachman (89) and interpreted in terms of interrupted twin chains held together by hydrogen bonds. Reexamination of these phenomena by Thomas (290), Doty (99, 291), and Shumaker *et al.* (271) more closely define this picture and establish that breaks in opposite chains, if separated by three to six hydrogen bonded base pairs, do not necessarily allow the dissociation of the resulting fragments. There is no evidence for the prior existence of interruptions in the chains to a degree greater than one in 3000. A slight preliminary attack by deoxyribonuclease, which produces random breaks in the two chains (89, 258, 271), in the earlier study (89) could explain the discordance between the apparent molecular weights of heat-denatured DNA, which is now shown (99) to retain its phosphodiester bonds intact. Although it has been shown that deoxyribonuclease will break approximately 25 per cent of the phosphodiester bonds of DNA, liberating the bulk of the substrate as low molecular weight polynucleotides (di- to octanucleotides) (277), light-scattering measurements indicate an apparent average molecular weight of 190,000 for the deoxyribonuclease-resistant mixture (258). The meaning of this is not apparent, but may be a result of simple physical aggregation.

A commentary on the use of physicochemical properties as criteria of

"native state" would seem to be offered by the observation of Rich *et al.* (261) that a mixture of polyadenylic and polyuridylic acids shows a large increase in viscosity, a decrease in absorption at 260 mμ and an x-ray diffraction pattern of its fibers "not unlike that of DNA."

The changes in viscosity, titration properties, and ultraviolet absorption of DNA after γ-irradiation are also consistent with the double-helix structure (70) [see also Butler in (C)], being explained on the basis of breaks at opposite positions in the two chains with rupture of cross-linking H bonds. The percentage of H-bond breakage, determined from the titration curves, can be related to the observed increase in ultraviolet absorption.

The importance of other substances on the sensitivity of DNA to radiation is shown by the effect of ferric ion, which at about 10^{-4} *M* seems to have sufficient effect to explain the difference between the sensitivities of DNA *in vitro* and *in vivo* (184) and molecular oxygen, which makes possible the formation of hydroperoxides from pyrimidine bases (270). The long half lives of some of these hydroperoxides may account for the previously observed delayed effects. The effects of ultraviolet and of x-radiation on nucleic acids and nucleoproteins are reviewed by Butler (42 and in C). Zamenhof *et al.* (313) have demonstrated the inactivation of transforming principle by ferrous iron and other auto-oxidizable substances, by mutagens (although not by the strongly mutagenic Mn^{++}), but not by urea or detergents. Inactivation occurs at doses of ultraviolet radiation lower than those that cause viscosity changes. The effects of radiation and peroxides on viral and bacterial functions linked to DNA are reviewed by Latarjet (in C).

Heterogeneity.—Although the physicochemical methods applicable to the study of large molecules fail to reveal any significant differences between the DNAs derived from normal and leukemic human leukocytes (251) or between those derived from normal and tumorous human spleen (250), the application of the chemical methods of differential extraction and of column chromatography continue to reveal heterogeneity within the DNA derived from one tissue. The sedimentation constants of DNAs extracted from thymus nucleoprotein by different salt and detergent procedures are significantly different when measured at the dilutions made possible by ultraviolet optics (274), and it appears that anionic detergents give preparations of higher yield and higher average sedimentation constant than those prepared by chloroform denaturation or water extraction. Inasmuch as the material unextracted by the latter two methods is of higher sedimentation constant, it appears that the observed heterogeneity is not produced by the mode of extraction. The advantages of xylene sulfonate as a protein denaturant (90) in the preparation of thymus DNA are described by Simmons *et al.* (276). DNAs of varying composition have been extracted from atypical epithelioma and Ehrlich carcinoma of the rat by varying salt and temperature conditions (174).

Both the T2r bacteriophage DNA (39), prepared by chloroform extraction or by osmotic shock, and a pneumococcal transforming principle (200)

have been shown to be heterogeneous in their rate of movement down cellulose-protein columns with increasing salt concentration. In the latter case, the biological activity per unit of DNA could be increased to a significant degree. Reproducibility of elution behavior was demonstrated in both cases by rechromatography of individual fractions.

The study of the change in base composition of the DNAs extracted by increasing salt concentrations has been extended (208) to the interesting case of wheat germ DNA, which has a high proportion of the fifth base component, 5-methylcytosine. The ratio of this component to cytosine varies over a wide range, being highest in the first fractions (lower NaCl concentration) and decreasing progressively in succeeding ones (higher NaCl concentration). The results achieved with increasing concentrations of salt for fixed lengths of time (208) seem quite comparable to those obtained by successive extractions with a single salt concentration (variable time) (214) and it appears that both are dependent on the same situation, namely, the varying electrostatic attractions between the different nucleic acids and proteins (or polybasic component). It is pointed out (208) that the chromatographic separations are also probably effected by this same principle, those with the stronger bonds being the slower moving. That fractions obtained by the successive extraction procedure may still be heterogeneous can be demonstrated by subsequent column chromatography of the individual fractions (122).

The varied location of RNA within cells and its metabolic activity predispose one toward the acceptance of chemical heterogeneity, which has now been demonstrated to occur in the RNA of rat liver microsomes under the influence of protein deprivation (306), and in the RNA of rabbit liver nuclei prepared by two different procedures (171). Inconsistency in the base composition and RNA content of such a primitive entity as the TMV particle, even when electrophoretically homogeneous, also leads to the conclusion of heterogeneous populations (67). However, the contamination of RNA by nucleases, which is an obvious possibility in those preparations arising from whole tissues (60), is also possible in TMV preparations, as Pirie has noted (248).

The establishment of heterogeneity by base analysis may be complicated by the widespread existence of polynucleotide phosphorylases (240), which may synthesize or degrade polynucleotide material during the course of isolation following cell rupture. Beers (18) has shown that *Micrococcus lysodeikticus*, in the course of lysis with lysozyme, can effect an interchange between acid-insoluble and acid-soluble nucleotide material, the direction of this interchange depending on the presence of inorganic phosphate and of ribonucleotide diphosphates. Such an interchange could markedly affect the integrity of polynucleotide material. In this connection, the rapid synthesis of diphosphates by mitochondria in the course of isolation should be noted (24).

Other aspects of heterogeneity in nucleic acids (base ratios, metabolism, biological properties) are dealt with subsequently.

Enzymes.—The action of pancreatic ribonuclease on cyclic pyrimidine nucleotides, a specific enzymatic action (227), has been developed into a spectrophotometric assay for the enzyme (262), to be added to the classical assays of Kunitz and the production of acid-soluble material from RNA, and the newer hydrolysis of synthetic 3′-pyrimidine nucleotide acyl or aryl esters. Richards (263) observes that the ratio of the first two of these activities is constant in the fragments isolated from column chromatography of ribonuclease that had been subjected to subtilisin digestion. A careful study by Davis & Allen (86) of the inhibition of ribonuclease action by products, various ions, ionic strength, and —SH inhibitors reveals a parallel behavior with respect to both types of synthetic substrates. The effect of divalent ions and of ionic strength are in some instances different from those reported by Dickman *et al.* (93) and others using RNA itself as a substrate, which may indicate (86) contamination of the RNA used or a differential solubility effect on the various substrates (e.g., the larger polynucleotides may be precipitated by the inhibiting ion) (93). Dodecyl sulfate and other anionic detergents and polyvalent anions, long known as protein denaturants (7) and nucleoprotein dissociators (281), are shown to be complete inhibitors of ribonuclease (74, 93, 138). Vandendriessche finds much less inhibition of the depolymerization of RNA (dilatometric measurement) than on the hydrolysis of the cyclic intermediates by poly acids of many kinds; the effects are reversed by polyamino compounds (294). Streptomycin is again shown to inhibit by combination with the phosphate of RNA (57, 88).

Both Allen and Dickman agree in finding no effect by —SH group inhibitors, in apparent contradistinction to Ledoux (192), who has demonstrated again that it is possible to inactivate solutions of ribonuclease with atmospheric oxygen, or other oxidants, to separate the inactive and active forms by column chromatography, and to reactivate the former with cysteine and reduced glutathione. Hakim observes activation of crystalline ribonuclease by glutathione (140a) and Weil and Seibles demonstrate ribonuclease inactivation by photo-oxidative destruction of three out of the four histidine residues; no —SH groups could be detected (305a). Ledoux claims that the failure of other investigators to demonstrate the reversible oxidative inhibition of ribonuclease is caused by its high oxidation-reduction potential ($E_0 = 0.64$ V). In addition, his means of assay (the classical Kunitz method) differs from those of the other investigators, who measured phosphodiester bond cleavage. Oxidized ribonuclease is generally ineffective *in vivo*, according to the experiments of Brachet and his colleagues (see next paragraph).

Ribonuclease, administered to living tissues, produces a variety of effects (33), not all of which may be traced to its nucleolytic action. Inhibition of cell division is noted in amphibian eggs (33, 195), ascites tumor (193),

amoebae (30), and tissue culture (55, 116). With radioactive ribonuclease, autoradiography indicates an attachment to mitochondria and a block in oxidative phosphorylation similar to that produced by dinitrophenol (195). It has been observed that ribonuclease can liberate acid phosphatase from rat liver mitochondria (4) and thus influence oxidative phosphorylation, which has been shown to be necessary for nucleic acid synthesis in homogenates (309). Growing onion roots confer upon solutions of ribonuclease with which they are in contact the property of degrading DNA [Feulgen stain (80)]; this may be related to the many observations of mitotic inhibition by ribonuclease that have also been ascribed to an inhibition of nuclear protein synthesis (33). The behavior of ribonuclease as a basic nonspecific protein toward nucleic acids under certain conditions (138) may be involved in its biological action; many basic proteins are toxic to amoebae (30). Brachet notes, however, that only ribonuclease has a pronounced effect on basophily (30).

At least part of the current disagreement concerning the activation or inhibition of ribonuclease may lie in the use of several different assays (see Schmidt in A): the classic Kunitz assay, measuring increase in absorbance at 330 mμ (cause not defined); the production of small (acid- and uranium-soluble) fragments and increase in volume from RNA preparations, or liberation of pyrimidine mononucleotides from their phosphodiesters (conversion of pyrimidine phosphodiesters to cyclic nucleotides); the conversion of cyclic to noncyclic pyrimidine nucleotides, measured by titration at pH 7 to 8, or by the action of phosphomonoesterases, or by the isolation of acyclic pyrimidine nucleotides (hydrolysis of cyclic pyrimidine nucleotides). Thus, there are at least two specific enzymic activities, each with its own sensitivities to extraneous materials and conditions (e.g., products, impurities, pH), residing in at least two separable protein species (140a), even in the crystalline ribonuclease preparations, which can undergo considerable degradation without apparent loss in "activity" (166a, 263). To these variables may be added the intrinsically basic character of the protein(s) that complicates the interpretation of its effects *in vivo* as well as upon biologically active nucleic acid-containing materials. It is not likely that the actions of ribonuclease will be fully understood or properly interpreted until these interrelated complexities are disentangled [see Hakim (140a)].

The role and importance of magnesium ion in deoxyribonuclease degradation of DNA is emphasized by Chargaff & Shapiro (53), who show that traces of the free ion (present in citrate but not in versene) are sufficient to allow the random diphosphoesterase action of the enzyme to begin. Partially degraded DNA, purified by deproteinization, centrifugation, and dialysis, can be further degraded by magnesium ion alone, as shown by the production of dialyzable fragments and increased phosphatase sensitivity. A nuclease with a mode of action apparently different from the standard pancreatic deoxyribonuclease has been isolated from *Micrococcus pyogenes* by Cunningham *et al.* (75). This enzyme is relatively heat stable and calcium

activated; it liberates a much larger proportion of the DNA as mononucleotides and appears to attack the end product polynucleotides of pancreatic deoxyribonuclease action. It is of considerable interest that the mononucleotides which are produced (some 27 to 30 per cent of the total) are insensitive to the 5′ nucleotidase of snake venoms and thus appear to be 3′ deoxynucleotides, not heretofore isolated from natural sources.

Another enzyme with unusual and unexpected action is a phosphodiesterase isolated by Davis & Allen (85) from beef pancreas that attacks only cyclic phosphates, converting them to the 2′-nucleotides exclusively. Inasmuch as the latter have not been found to occur naturally (except in TPN), the biological significance of this enzyme is not immediately apparent.

The use of an enzyme for structural investigations implies an understanding of its chemical specificities, among which must be included reversibility and transferring (metathesis) activity. The action of the potent and useful phosphatase of prostate has been shown to be reversible (268). At high concentrations of cytidine and inorganic phosphate, all three cytidylic acids (5′ is favored) and two dinucleotides are formed. Ethyl phosphate is a more effective donor; in this case, the action could appear to be that of a transferase. That transferase and hydrolytic properties are not necessarily different aspects of a single enzyme is shown by the separation of the nucleotide transferase and phosphatase activities of carrot extracts (293). In this case, the transfer of phosphate (from phenyl phosphate) also favors the 5′ position.

Intramolecular structure.—The establishment of a 3′,5′ structure for RNAs and the elimination of the need for referring to these as pentose nucleic acids has been described in previous reviews (5, 37). The clarification of certain aspects of RNA structure and the attack on the remaining problems of sequence and end groups will concern us here.

The only substantial evidence advanced for branching in RNA via triply esterified ribose was the appearance of relatively large amounts of pyrimidine nucleoside diphosphates by the action of snake venom diesterase on various RNAs (63) and the isolation of ribose from RNA methylated by silver oxide and methyl iodide (6a). Reexamining the first reaction, using yeast RNA prepared so as to eliminate ribonuclease degradation or the presence of ribonuclease in the product (74), Crestfield & Allen (73) obtained roughly equivalent amounts of all four nucleoside diphosphates in a yield consistent with their origin from the ends of single, unbranched chains, roughly 75 nucleotides in length. The pyrimidine members were increased when ribonuclease was present (73). By methanolysis degradation, in which end groups appear as simple mononucleotides, the same conclusion is reached by Lipkin *et al.* (206, 287), as to the structure of the calf liver RNA used in the older experiments, whereas commercial yeast RNA gave considerably larger amounts of unmethylated mononucleotides, particularly in the pyrimidine members, these constituting proof of prior degradation. The conclusion seems inescapable that the RNA preparations used earlier (63) were

contaminated with nucleases that produced shorter chains with pyrimidine endings during the diesterase degradation. The isolation by Kaplan & Heppel (167) of a ribonuclease from spleen with identical specificities but differing pH optimum and chromatographic behavior from pancreatic ribonuclease underlines this possibility. The assumption of complete methylation of RNA by silver oxide and methyl iodide has been shown to be invalid (9a). Therefore, there is no need at this time to invoke branching in RNAs to explain the appearance of the diphosphates in the one experiment or of ribose in the other (60).

The identification of the *a* and *b* purine nucleotides as 2′ and 3′, respectively, has been accomplished by both synthetic and degradative procedures (5, 37), whereas that of the pyrimidine isomers has hitherto rested on physicochemical properties (5). Reduction (61, 299) and hydrazinolysis (11) of the pyrimidine *a* and *b* nucleotides has made possible the isolation and identification of ribose 2- and 3-phosphate, respectively. Uridine-2′-phosphate has been synthesized unambiguously and shown to be identical with uridylic acid *a* (38). Thus the identification of all of the isomers has been accomplished by a variety of means.

Sequence.—Incomplete hydrolysis of RNA by bismuth hydroxide (96, 97), and HCl (62) has been exploited to yield mixed dinucleotides or dinucleoside phosphates that have been examined for the presence or absence of the sixteen possible sequences. Although some (ribonuclease-sensitive ones) were at first reported as missing (97), subsequent work reveals that all are present in yeast RNA (62, 96). The migration of unhydrolyzed phosphodiester bonds during acid (36) or basic ion-exchanger (95) treatment, which has significance in this type of investigation, has been observed.

Acid hydrolysis of thymus DNA to investigate sequences has been exploited in a variety of ways. Mercaptalation (173), mercaptoacetylation (164), acid treatment (64), or the diphenylamine reaction at 30° rather than 100°C. (41), all appear to proceed via the hydrolysis of purine residues as a first step, since this occurs at a rate some 200 times that of phosphodiester cleavage (291), thus permitting the isolation of purine-free macromolecules (164). The subsequent degradation of these products (apurinic acid, mercaptalated or mercaptoacetylated DNA) can be accomplished by alkali (165, 173), in which the action proceeds via cyclization between 3′ and 4′ or 4′ and 5′ hydroxyls (165), or by further acid treatment, causing elimination, to yield the pyrimidine nucleotide sequences of the original DNA. The purine sequences should be related to these by the complementary nature of the molecule (305). Purine-pyrimidine sequences can be established only with the aid of nucleases. From the work of Sinsheimer, it appears that there are wide variations in the relative amounts of certain purine-pyrimidine sequence isomers in the DNA of both thymus (277) and bacteriophage T7 (215).

Nucleoproteins.—The question of constancy of composition of deoxynucleoproteins has received considerable attention. Those isolated from the

sperm of several species of fish and from some mammalian species tend, when purified, to ratios of phosphorus to arginine of 1 to 1.5 (107, 109, 178, 202, 297), whereas those from somatic tissues tend toward ratios of 4 to 5 (178, 297). The latter, upon repeated extraction, appear to lose more lysine than arginine, with a decrease in the phosphorus to arginine ratio (87, 214, 297). Preparations that are half DNA and half protein, of uniform physical properties (100), and yield DNA of high molecular weight have been obtained from calf thymus. The constancy of the ratios of lysine to arginine to phosphorus (0.47/0.30/1) in purified thymus nucleoprotein preparations is noteworthy (87). Lison (209) notes a rise in arginine content of the maturing sperm of the rat, with a simultaneous decrease in basophilic character, a matter of particular concern in histochemical observations.

The bulk of cellular RNA is contained in the microsomes (Hogeboom & Schneider in A, 54). The RNA-protein particles liberated from rat liver microsomes by deoxycholate contain about half of the microsomal RNA and are themselves about half RNA and half protein (211) with a molar ratio of amino acid to nucleotide of approximately two (52). This ratio is maintained throughout various mild reprecipitations, but about half of the protein is solubilized by papain, or by alkali after trichloroacetic acid precipitation (52). A particle with a similar RNA to protein composition has been found in yeast (51).

Methods.—The extraction of RNA from tissues (66, 176) or TMV (130, 131, 272) with phenol appears to offer the multiple advantages of precipitating protein and DNA, inhibiting ribonuclease, near quantitative yield, and a highly polymerized product (66, 272). DNA from those bacteria susceptible to lysozyme can be obtained in high yield and fibrous form by the controlled use of this enzyme (18, 243).

The ease of hydrolysis of RNA in alkali continues to be most useful for the analysis of RNA and its separation from DNA, particularly as a prelude to separation of nucleotides and bases by chromatography (218) or after a hot salt extraction that, even in plant tissue (223), can be made essentially quantitative. Some of the non-nucleotide phosphorus compounds that contaminate the resulting ribonucleotides have been identified as inositol and serine phosphates, and peptides are also present (161). Much stronger treatment with alkali is required to affect DNA to any degree (160). Although the presence of alkali-resistant fractions of RNA other than the known nucleotides has been reported (252, 280), it has yet to be demonstrated that these affect significantly the use of this method for the recovery and analysis of the base components of RNA. The finding of such unexpected constituents in digests of RNA or DNA preparations, or of excess amounts of phosphate (35), is not proof that these were at any time covalently bound into the polynucleotide structures. It is well to beware of formulating structures or biochemical mechanisms on the basis of analytical correlations that may be adventitious, circumstantial, or fortuitous.

Analytical methods for nucleic acids and their components, in particular

those involving paper chromatography, are explicitly set forth by Markham (220). Trifluoroacetic acid is recommended for the hydrolysis of RNA or DNA to the respective bases in place of perchloric or formic acid, particularly as a prelude to paper chromatography (103).

Concentration in Cells and Composition

DNA per nucleus.—In a comprehensive review of analyses of nuclei for DNA, Vendrely (296) emphasizes again the concept of the constancy of the amount of DNA per chromosome within a given species and the use of this figure as a parameter in comparative biochemistry. The first of these generalizations has received renewed confirmation and extension by histophotometric studies of guinea pig (81), rat (104), and mouse (168) diploid nuclei, and by chemical assay of Yoshida sarcoma cells (127). Changes in chromosome number among such related groups as the ass, horse, and mule (298), sublines of two mouse ascites tumors (65), and various tumors and their normal counterparts (230), are paralleled by the amount of DNA per nucleus. The dependence of DNA content on the degree of polyploidy is shown in human liver, which exhibits nuclei of up to octaploid value with increasing age of the individual (284), and in a haploid-diploid species with still higher degrees of polyploidy (239).

Most of the recorded cytochemical observations of this type utilize the Feulgen reaction, the advantages of which over basophilic staining are emphasized by Lison (209). Although the method affords the generalizations noted—the variations of individuals but the constancy of the mean—variations beyond what may be ascribed to technique are still to be noted (210) and the Feulgen reaction indicates low (185) or no (222) DNA in unexpected circumstances. The absence, by Feulgen test, of DNA in the mature sea urchin egg (222) does not seem to accord with its normal diploid behavior when the same test is made after fertilization (228). The possibility that different assay methods may not give concordant results, perhaps because they measure different materials, is indicated by the apparent existence of 10^3 times the haploid amounts of DNA in cricket (102) and frog (213) eggs when thymine and microbiological (154) assays, respectively, are used. In the latter case, the Feulgen reaction does not corroborate the high figure, perhaps because the microbiological assay measures deoxyribonucleosidic material other than DNA. Nor does the finding of polymerized DNA extracellularly necessarily imply an extracellular origin (44).

The amount of DNA found in mature fish sperm by Felix *et al.* (108) accords fairly well with the amounts in various other spermatozoa (102, 108, 296). The sperm deoxyribonucleoprotamine has a measured volume equivalent to the volume of the sperm head itself (108), which lends weight to the argument that the amount of DNA in mature sperm represents the haploid amount of the species in question (81, 102, 108, 239, 296). The amount of DNA per nucleus in marsupials is the same as that of Eutherian mammals

and considerably different (three times) that of birds, which accords with the relative evolutionary position of these species (260).

The generalization of the constancy of mass of DNA per chromosome, just discussed, is further confirmed by studies of its variation during mitotic and meiotic cycles. In mammalian (76, 105, 146), ascites (156), and various invertebrate (82, 134, 228) cells, it is shown that DNA synthesis begins in interphase, reaches the required value (allowing for degree of ploidy) before metaphase, and is divided among daughter nuclei (and polar bodies) at metaphase (see Howard in C). In the developing sea urchin embryo from fertilized egg through pluteus stage, all cells maintain the diploid amount (228). In synchronously dividing *Escherichia coli* (10, 229) and *Salmonella typhomurium* (188), and in chick fibroblast tissue cultures (115), DNA per cell doubles before division. The cell proliferation in the vaginal epithelium of the rat in estrus is also accompanied by an increase in its DNA (266); changes in DNA in irradiated Walker carcinoma of rats (a decrease) (145) and in the livers of alloxan diabetic rats (an increase) (94) are limited to the very small DNA-1 fraction (20). DNA-1, not detectable in the livers of fully grown rats (20) increases markedly in regenerating liver (119). However, alloxan diabetes does not, like regeneration, increase the mitotic rate (94).

These observations serve to reinforce the concept and general use of DNA as an indicator of numbers of nuclei and therefore as a more meaningful baseline than cell mass for expressing the analytical and metabolic properties of cells (135) (see following sections).

Stability of DNA.—Tracer studies reinforce the generalization that DNA is synthesized only in accordance with the needs of chromosomal multiplication and is otherwise metabolically inert, failing to show turnover in the absence of net synthesis. Continuous exposure of rats to a uniform $C^{14}O_2$ atmosphere, which labels all cell components as these are formed, shows that the percentage increase in the liver DNA is similar to the percentage increase in liver weight (i.e., specific activity ratio of DNA to RNA is one half for a doubling in weight); the average life span of DNA is estimated to be at least 150 days, in contrast to 3.5 to 6.5 days for RNA half-life (285). Weanling rats, labelled with P^{32} via the milk of the mother, retain the label in the DNA but lose it from the RNA (175). The low rate of incorporation of P^{32} into the DNA of adult rat liver is increased when the mitotic index is raised by a tumor elsewhere in the animal (234), and by hematopoietic regeneration (233). The rapid growth of tumors in comparison to normal tissues is paralleled by an increased incorporation of P^{32} in DNA (234).

The most recent and careful quantitative studies of the rate of P^{32} incorporation into the DNA of regenerating rat liver indicate that the total amount of DNA synthesized is equal to the new DNA that appears (that is, the old DNA is retained) (79, 162). From these studies it again appears that the peak of synthesis occurs prior to the peak in mitotic index (162, Holmes

in C). A similar conclusion is drawn from the small extent of incorporation of P^{32} into the DNA of several mammalian tissues *in vitro*, where cell division does not occur (155, 241), and from the higher incorporation into RNA relative to DNA *in vitro* compared to *in vivo* (241). Incorporation of precursors in proportion to mitotic index, cell number, or DNA doubling is also observed in the developing chick embryo (120, 221), Ehrlich ascites cell (Forssberg in C), and *Tradescantia paludosa* inflorescences (236).

Thus the evidence continues to support the older hypothesis of DNA as a substance formed only incidentally to cell division and metabolically inert thereafter.

RNA content.—Considering the DNA of a tissue as an indicator of cell number, and increases in DNA to be indicative of proliferation, changes in the ratio of RNA to DNA in various biological states become measures of RNA synthesis or degradation in these states. In Palladin's studies of the developing rabbit brain (242), an apparent drop in RNA concentration from embryo to adult of 416 to 166 mg. per cent becomes, owing to a still greater drop in DNA, an increase of threefold in RNA/DNA. Hughes (157) finds that the RNA density in various nerve cells is correlated with neuronal differentiation in the chick embryo. The large increase in DNA of rabbit ganglion fibers as a result of chromatolysis, probably owing to cell proliferation, is matched by a similar increase in RNA, whereas stimulation of such fibers causes a fall in RNA/DNA to about one-eighth its previous value (45). In the Lanschütz ascites tumor, the 50 per cent fall in RNA/DNA (DNA per cell was constant) is correlated with the decrease in growth rate (196).

Current interest in adrenal function has given rise to several investigations of the behavior of the nucleic acids of this gland and other tissues under the influence of the hormones involved. In rats, administration of ACTH (217) or its appearance in conditions of stress [e.g., cold (3, 238) or prolonged protein fasting (216)] causes a rise in the RNA of the adrenal glands; DNA also rises (3, 238) in these conditions. There is a decrease in the RNA of other tissues (216), which is reflected in the RNA of all subcellular constituents (186). Adrenalectomy causes an apparent increase in the synthesis of supernatant RNA of rat liver in opposition to the decrease in microsomal RNA, whereas hypophysectomy (no ACTH) reduces the synthesis in all fractions (259). The elimination of ACTH by hypophysectomy or the administration of cortisone produces a fall in adrenal RNA and DNA (217, 273) and inhibition of P^{32} incorporation into thymus and spleen DNA, paralleling the inhibition of cell proliferation (56). Human adrenal material obtained at autopsy or operation shows a similar pattern of change (83, 286). The tendency for adrenal RNA and DNA to increase with increased adrenal function is interpreted (217) as an increase in the mass and number of cells.

Base ratios: DNA.—The generalization that forms a cornerstone of the Watson & Crick structure (305) and which requires that in DNA in terms of moles, G is equivalent to C+MC, and A to T (Chargaff in A, 37, 108),

has been examined in a wide variety of species and variants. Of six plant species only one showed both A=T and G=C+MC (289); MC, to the extent of one-tenth of the total base content, was found in rye (289). Not all analyses of mouse liver and kidney (269), or of ascites tumors (174, 269; but cf. 204) show the usual correspondence (Chargaff in A, 108). Seven species of fish sperm nuclei gave A/T=1.04 to 1.12 and G/C=1.0 to 1.1 (107, 108, 109). (These are the same specimens that showed phosphorus to arginine ratios of 1/0.95 in their nucleoprotamine forms.) However, bacteriophage T7 (215) and sixty strains of bacteria (198) show A/T=G/C=1.

The use of A+T/G+C ratio as a species parameter is encouraged by the finding that, although their ratios varied from 0.4 to 2.7, the actual values found by Lee *et al.* (198) in 60 strains of bacteria were characteristic of species and of family classifications. Variations in the metazoöns examined (fish, echinoderm, insects, birds, mammals) varied from 1.2 to 2.2 (107, 197). Bacteriophage T7 had a ratio of 1.08 (215), T2r a ratio of about 2 (39).

The fractionation of DNA by solubility properties in general yields materials with ratios of A/T=G/C=1 but of differing A/G ratio. Bacteriophage T2r DNA has been resolved on cellulose-histone columns into two fractions with ratios of 1.90 (30 per cent) and 2.15 (70 per cent), respectively (39), and thymus (214) and wheat germ (208) nucleoproteins, fractionated by repeated salt extractions, lose material of high G and C content in the earlier or weaker fractions. Fractions prepared from atypical rat epithelioma and Ehrlich carcinoma, however, exhibited irregular base ratios (174). From these and other experiments (174), it is apparent that the base composition of a DNA preparation may be dependent on the relative solubilities of its component species.

Base ratios: RNA.—Although in RNAs there seems little evidence for the correspondence noted in DNAs, there is a tendency (Magasanik in A), for the 6-keto bases to equal the 6-amino bases (i.e., G+U=A+C). This is observed in both normal and streptomycin-resistant strains of *Sarcina lutea*, where the ratio of G to A in the RNA approaches two and in DNA is almost three (103). A ratio of 1.12 for G+U/A+C observed in Ehrlich ascites cells is not changed by encephalomyocarditis virus infection, which doubles the rate of P^{32} incorporation into both RNA and DNA (204), but is significantly altered (C rises, G falls) when the spread of the tumor is inhibited by ribonuclease (193). A DNA-like correspondence (G/C=A/U=1; A+G=C+U) is reported for human pancreas by Filipowicz *et al.*, who make the same claim for ox pancreas RNA (114). However, significant ratios are only occasionally observed in beef or calf pancreas (Magasanik in A, 176), possibly caused by the rapid action and resistance to denaturation of the ribonuclease of this tissue. The susceptibility of RNA to enzymic and chemical degradation during isolation may be the cause of the wide variations in base ratios reported for the same tissues by different investigators; the lack of correspondence in composition from one preparation to another from the same initial tissue extract (307), or among different purified preparations of one

strain of TMV, lends weights to this argument. The variation of base distribution in the liver microsomes of protein-fasted rats, noted by Weill *et al.* (306), indicates that the observed RNA composition may depend even on the nutritional state. The further possibility of disturbances caused by the synthetic or degradative action of polynucleotide phosphorylase during the isolation has been mentioned (18).

Metabolism

Nucleoside 5′-phosphates as immediate precursors.—Some of the implications of the discovery by Grunberg-Manago, *et al.* (139, 140) of an enzyme system, apparently of widespread occurrence (17, 18, 240), which forms 3′, 5′-polynucleotides from ribonucleoside 5′-pyrophosphates, have been discussed by Carter (43). Chief among these is to support the conclusions drawn from the earlier tracer experiments that the 5′-nucleotides are precursors of the nucleic acids *in vivo*. The decay of P^{32}-labelled fractions also indicates that adenylic acid rather than inorganic phosphate satisfies the criteria of a nucleic acid precursor (13). The presence of the required ribonucleoside 5′-pyrophosphates (e.g., ADP) in the acid-soluble fraction of many tissues has been amply demonstrated. The pyrimidine deoxyribose analogues have been found in thymus (253). It is of interest that 5′-ribonucleotides are present in nuclei isolated in a nonaqueous medium (6, 170). Such nuclei also contain an enzyme that splits polyadenylic acid to 5′-ended nucleotides and polynucleotides (151). The same nuclei isolated in aqueous media are rich in triphosphates, indicating the presence of phosphorylating enzymes (6). Whole tissues (78) and certain cytoplasmic fractions can rapidly phosphorylate nucleoside-5′-phosphates to di- and triphosphates (152), even in the course of isolation of the fractions (24).

More direct proof for the incorporation of 5′-deoxynucleotides intact into nucleic acids arises from the simultaneous administration of 5-bromouracil and inorganic P^{32} to growing *E. coli;* the 5-bromouridylic acid isolated from DNA after a short period of growth had a threefold higher specific activity than the other four nucleotides (255). Enzyme fractions from this organism have been shown by Kornberg *et al.* (181) to be capable of converting thymidine to the 5′-nucleotide, phosphorylating this to the triphosphate, and polymerizing the latter (but not the diphosphate) to an acid-insoluble product digestable by deoxyribonuclease. Some DNA or DNA fragments and ATP are required and the rate of incorporation (both C^{14} and P^{32} labelled thymidine 5′-phosphates have been used) is increased markedly by the presence of the other three deoxynucleoside triphosphates. A reverse effect (inhibition of thymidine incorporation into thymus nuclei *in vitro* by other deoxynucleosides or deoxynucleotides) is observed by Friedkin (122).

Although several attempts to demonstrate the incorporation of 5′-ribonucleotides as such into RNA were unsuccessful, owing to dephosphorylation in transit (201, 205, 264, 265), the use of fortified cell-free homogenates has

permitted this demonstration. Thus AMP^{32} is shown to be incorporated (to the extent of 1/6000) (132, 149), and to be recoverable as such, with essentially no activity in other nucleotides, by the action of venom diesterase (149). Alkaline hydrolysis gave cytidylic acid which contained nearly all the label. That cytidylic acid should be singled out as the specific linkage point indicates a nonrandom mechanism or choice of molecular species.

The transfer of phosphate from the 5′ position of the precursor nucleoside to the 2′ or 3′ position of the adjacent nucleoside when RNA is hydrolyzed by alkali means that the specific activities of the phosphate of alkali-produced nucleotides are not necessarily those of the precursors. Therefore, the oft-repeated experiment (29, 84, 232, 308, Smellie in A) of administering inorganic P^{32} and determining the phosphorus activities of the RNA nucleotides after alkaline hydrolysis cannot be expected to indicate the metabolic behavior of precursor nucleotides. It is now to be expected that the latter may be heterogeneous and, along with nonuniformity in metabolic behavior and in constitution of the various RNAs, give rise to heterogeneity in phosphorus specific activities among the nucleotides in RNA. This will be reflected in the 2′- and 3′-nucleotides of alkaline hydrolysis, but with varying degrees of perturbation. It is probably of significance, in this connection, that the order of specific activities obtained in experiments of this kind bears an inverse relation to the frequency of occurrence of the individual bases in the RNAs in question (Smellie in A). In other words, the total labelled precursors that attach to each kind of purine or pyrimidine nucleoside in RNA is somewhat proportional to the amount of each which is found in the RNA.

Inhibition of metabolism.—In view of the large number of synthetic steps from small remote precursors to the immediate precursor of the nucleotide (43), it is apparent that unequal degrees of incorporation of such small precursors even into one immediate precursor are to be expected. Thus each remote precursor may give a different turnover figure, dependent on the particular efficiency of those steps governing its incorporation. The variation in enzyme pathways even in normal states is shown by the variation in utilization of standard precursors (e.g., formate, glycine, CO_2, adenine) in bacteria (26) and in rat tissues (1, 219, 235). In abnormal states, such as produced by vitamin E deficiency (98), tumor presence (126, 234), Aminopterin (246), and radiation (155, 162, 241, 246), heterogeneous incorporation is observed, but it is not always clear which enzymic steps are perturbed. It has occasionally been possible to show a direct relation of precursor to product. Thus Swick *et al.* show that the CO_2 that forms the C_6 position of the purines is in metabolic equilibrium with urea, and that the glycine that forms positions 4, 5, and 7 of the purines is in metabolic equilibrium with liver protein glycine (specific activities in each case are identical) (285). The importance of thymidine rather than thymine as the source of DNA thymidine in animal tissues is shown by Friedkin *et al.* (120, 121) and Reichard (257), but both are ineffective in *E. coli* (275). The inhibition of the conversion of de-

oxyuridine to DNA thymidine in bone marrow cells by Aminopterin, without influence on thymidine utilization, seems to pinpoint one locus of the inhibition by this substance (121).

A controlling role of vitamin B_{12} upon the synthesis of deoxyribose is shown by the finding of Downing & Schweigert (101) that *Lactobacillus leichmannii*, in the absence of B_{12} incorporate the sugar of added thymidine without dilution, whereas a large dilution is noted in the presence of B_{12}.

Comparisons of the relative rates of incorporation of single precursors into the nucleic acids of tumor tissues and normal tissues in the same animal indicate no profound differences with the single exception of guanine, the incorporation of which is relatively decreased in the tumor tissue RNA (8, 9, 22, 23). A similar discrimination is noted in the tissues of hepatectomized animals which incorporate more of many precursors but not of guanine (9). The lag in the incorporation of P^{32} into either position (232) adjacent to guanosine in the RNA indicates a slower turnover of both GMP and those phosphates to which it couples. It is also of interest that the polynucleotide phosphorylases from a variety of sources find it difficult to polymerize guanosine diphosphate (17, 240).

The well-known inhibition of nucleic acid synthesis by ionizing radiation has been the subject of innumerable investigations, none of which have indicated the step or steps primarily affected (see Forssberg, Holmes, Howard in C). Preliminary attempts to find an increase in acid-soluble precursors after irradiation, on the assumption that these might increase as the result of a block in the polymerization step, have been reported, with varying results. Essentially no changes are found by Bećarević *et al.* (16) in the acid-soluble nucleotide content of spleen after x-irradiation, whereas Bishop & Davidson (25) find a sharp temporary rise in the appendix, a lesser one in thymus.

Analogues.—Incorporation of unnatural analogues of various purine and pyrimidine bases into RNA and DNA has been established for both bacterial and virus systems [see review by Matthews & Smith (225)]. Inhibition of DNA synthesis and participation in cell functions do not always follow. Thus, in 15T⁻ *E. coli*, incorporation of 5-bromouracil (in place of thymine) leads to death after one division (59), whereas *E. coli* B shows a change in colony morphology but none in reproduction or DNA content (312), and the analogue appears in the DNA of nondividing as well as dividing cells (314). It has been isolated, as its nucleoside, from the DNA of *Enterococcus stei* (308a). 8-Azaguanine is incorporated into turnip yellow mosaic virus with reduction in infectivity (224). In *Bacillus cereus* RNA, it is found largely at the ends of the polynucleotide chains; it appears as if the further elaboration of the chain is thus inhibited (226). When thiouracil is incorporated (to the extent of 20 per cent of the uracil) into the RNA of *Bacillus megatherium*, a linear growth rate is observed, that reverts to normal logarithmic growth, after a lag, when uracil is restored (143).

The varied growth and other physiological responses to these and re-

lated analogues (128, 304, 310) do not encourage the hypothesis that their actions are caused solely by incorporation into nucleic acids. Indeed, it is shown that even the normal bases, in concentrations ten times those of the analogues, can also produce preprophase inhibition and chromosome breaks followed by death in plant cells (92). The mutagenic effects of certain substituted xanthines are not to be ascribed to incorporation into nucleic acids (179). Some analogues have been shown to have effects on the synthesis of purine coenzymes (110), upon enzymes involved in purine synthesis (136), purine nucleoside phosphorylase (180), and xanthine oxidase (106), all of which could influence or cause the observed morphological and physiological effects. Welch has reviewed (in B) the interferences of analogues, antimetabolites, and other substances in the biosyntheses of various nucleic acid intermediates.

Subcellular loci.—The independence of RNA synthesis in nucleus and cytoplasm has been demonstrated by microdissection techniques in both the amoebae (249) and in the alga, *Acetabularia mediterranea* (32, 295). In the latter species, upon enucleation, the rate of synthesis for both RNA and protein increases at first, and then continues at normal rates for at least several months. Oxidative phosphorylation, a necessity for nucleic acid synthesis as demonstrated in subcellular systems (19, 132, 149, 181, 254, 309), continues unabated (32). The apparent independence of cytoplasmic RNA formation does not seem to exclude movement of RNA phosphate from labelled nuclei into unlabelled cytoplasm of amoebae (131), although with isolated rat liver nuclei, the transfer of phosphate proceeds only in the other direction (212). This transfer, studied by Logan & Smellie (212), is enhanced by cytoplasmic particles and by two nondialyzable fractions from cell sap that show relative specificities toward RNA and DNA.

Heterogeneity of metabolic behavior and the greater metabolic activity of nucleolar RNA than of other nuclear RNA (and thus of all other cellular RNA) has been observed in a variety of species by the isolation of nucleoli (144) and by autoradiography (113, 288), and is in accord with the demonstration that rabbit liver nuclei contain two chemically separable RNA fractions of differing composition and metabolic activity (171). A remarkable discordance in metabolic behavior is observed in phage-infected *E. coli*, where a particulate fraction which contains less than 5 per cent of the bacterial RNA incorporates administered inorganic P^{32} to a specific activity three times the supernatant RNA (one-third of cellular RNA) and nine to sixteen times that of the RNA-rich particles (two-thirds of cellular RNA) (303).

Biological Involvements

Protein synthesis.—The case for a direct dependence of protein synthesis upon nucleic acids has rested almost exclusively on their juxtaposition or superimposition in time and space and on the parallelism in their responses to various inhibiting or stimulating agents. Protein formation has been meas-

ured as labelled amino acid incorporation into peptide linkage, as net protein increase, or as the appearance of a specific protein (adaptive enzyme or virus particle). The involvement of nucleic acids rests on the amounts demonstrable at the sites of protein formation and their changes during protein synthesis, or on their metabolic activity as shown by the incorporation of one or another precursor during such synthesis. The subject has been reviewed in general by Brachet (in A and C), with emphasis on the work with adaptive enzymes in bacteria and yeast by Gale and by Spiegelman (in B and C), and with respect to subcellular systems from mammals by Zamecnik (in C, 311), and by Borsook (28). The evidence to be reviewed does not differ substantially from that presented in the foregoing articles.

Comparing basophily and amino acid incorporation in various organs of the mouse, Ficq & Brachet demonstrate an exact correspondence (112). In a similar study of the course of oögenesis in amphibians, Ficq (111) observes that the site of maximum amino acid incorporation shifts as RNA concentration shifts within the cell from the cytoplasm (younger) to nuclei (middle aged) to nucleoli (older oöcytes).

The great intensity of amino acid incorporation by the RNA-rich microsomes (311) is confirmed and extended in rat liver (158, 259), chick liver (158), and rabbit reticulocytes (256). Although it is often taken for granted that nuclear protein synthesis is more intense than cytoplasmic, in the mouse the liver is the only tissue to show this (112); in mouse pancreas, trypsinogen, and chymotrypsinogen in the so-called supernatant fraction (usually regarded as slow compared to microsomes) are synthesized at rates exceeding that of the microsomal protein (77). Thus variation with tissue heterogeneity within a tissue fraction may invalidate generalizations drawn from complex fractions of single tissues.

The site of the protein synthesis in the microsomal system studied by Zamecnik *et al.* (172, 311) has been further defined by the use of deoxycholate which liberates particles containing equal quantities of RNA and protein, the latter displaying a more rapid rise in specific activity than the bulk of the microsomal protein removed by the deoxycholate (158, 211). Synthesis of the specific protein, amylase, in a granular and in a soluble system prepared from pancreas (283) and of the adaptive enzyme, β-galactosidase, in protoplasts from *B. megatherium* (187) have been reported. As with the microsomal system, these syntheses are sensitive to ribonuclease. However, ribonuclease does not interfere with the incorporation of amino acids into protein of isolated nuclei (6); the protein attached to DNA is the most active and the synthesis is destroyed by deoxyribonuclease.

The action of ribonuclease, used as a means of degrading RNA and interfering with RNA synthesis, has been correlated with diminished protein synthesis in a variety of situations. Portal resorption of ribonuclease causes a disappearance in the RNA of parenchymatous cells of liver and a parallel drop in plasma albumin, but a rise in certain globulins (237). That the action of ribonuclease *in vivo* is not simply nucleolytic is shown by experiments

of Ledoux (194) and Brachet (31) on ascites cells and onion root tips, respectively, from which it appears that ribonuclease acts first as a basic protein, many of which stop amino acid incorporation. The initial rise in RNA and protein in ascites cells, later reversed, are explained (194) as independent accelerations of RNA metabolism (labelled adenine uptake) and pinocytosis. Brachet & Ledoux (33) indicate the wide variety of responses to ribonuclease of various systems, ranging from no penetration (yeast, neurospora, ciliates) to rapid penetration with RNA reduction (amoebae, ascites, starfish eggs, rat liver slices) to penetration without change in RNA (onion). Although ribonuclease prevents the formation of penicillase if present during the induction period, a prior treatment, followed by removal of the ribonuclease enhances the rate of enzyme formation above that of controls (182). The protoplasts of *B. megatherium*, whole cells of which (strain M) show parallel stoppage of RNA synthesis (but not turnover) and incorporation of amino acids into protein when treated with ribonuclease (137), lose their ability to synthesize the adaptive enzyme, β-galactosidase, and to support phage growth (34, 187). Ribonuclease removes RNA but also fragments the protoplasts. Deoxyribonuclease is usually ineffective (34, 187).

Inhibition of nucleic acid synthesis in yeast by phosphate deprivation does not prevent a doubling of the protein content (267), and glycine incorporation into the protein of ascites cells is not inhibited when incorporation of glycine into purines is stopped by azaserine (199). Methionine deprivation in *E. coli* K12W6 permits an 80 per cent increase in RNA but little or no protein or adaptive enzyme synthesis (27); unlike strain $15T^-$ the cells remain viable during this period. Prevention of DNA synthesis in strain $15T^-$ (absence of thymine) or yeast (by ultraviolet radiation) does not prevent an increase in both RNA and protein (141) in a constant ratio (21). In the presence of chloromycetin, which inhibits both protein and RNA synthesis in amino acid-deficient media (but only the former in complete media), Pardee observes that traces of amino acids will restore the ability to form RNA; removal of the chloromycetin from this system gives a lower degree of RNA synthesis (244). A similar situation obtains with phage DNA synthesis in *E. coli* (see *Bacteriophage infection*).

Inhibition of adaptation in yeast by ultraviolet radiation inhibits the incorporation of adenine (15), glycine and phosphate (141) into RNA, but the adaptation is seemingly the more sensitive (15). Creaser (72) demonstrates that inhibition of RNA synthesis by the incorporation of 8-azaguanine into the RNA of *Staphylococcus aureus* blocks the formation of adaptive enzymes but not of constitutive enzymes, even when the character of the same enzyme (catalase) varies from one strain to another. When the block is reversed by guanine, RNA and DNA synthesis (C^{14}-uracil incorporation) is markedly increased. These findings are in keeping with the general conclusions from earlier work with disrupted cells (Gale, in B and C) that preformed RNA is necessary for the synthesis of constitutive enzymes but that RNA synthesis is necessary for the appearance of adaptive enzymes. That

the RNAs involved may be qualitatively different is indicated by Chantrenne's observations that in catalase-adapting yeast, adenine incorporation not only exceeds that of the controls (even when the enzyme formation is blocked by an amino acid inhibitor) but is heterogeneous in specific activity among subcellular (50) or ammonium sulfate (49) fractions.

The ability of RNA, isolated from *B. cereus* 569 H containing penicillase as a constitutive enzyme, to cause the appearance of penicillase in strain 569 which does not without addition of the normal inducer, is reported (182). Similar results, which appear to be unpredictably variable, are reported for the induction of β-galactosidase in *B. megatherium* (159). The sensitivity of this phenomenon to ribonuclease is noted (182) but the variability in reproducibility and the as yet ill-defined nature of these and most other RNA preparations warrant reservations in assigning the cause.

Apart from the basic concept of such work, which relates RNA only as a macromolecule to protein synthesis, there is also the possibility that interrelationships can exist at lower levels of complexity. Combinations of nucleotides and amino acids have been detected in various tissues (245, 252; see Smellie in A) and amino acid-nucleotide compounds, possible protein intermediates, can be utilized for ATP formation by *E. coli* enzymes (91). At least one of the specific amino acid incorporation factors, first found in whole RNA preparations and then in ribonuclease hydrolyzates of them, has been shown not to be a nucleotide (Gale, in B). The accumulation of UDP-amino acid compounds in penicillin-inhibited *S. aureus* (245) and the demonstrated sensitivity of uracil compounds to ultraviolet radiation, together with the dependence of protein synthesis on energy metabolism, involving nucleotide coenzymes, would seem to permit consideration of protein and nucleic acid interrelations in terms of lower levels of molecular complexity.

Bacteriophage infection.—The changes in nucleic acid and protein metabolism consequent upon bacteriophage infection have been further explored by use of amino acid-deficient mutants and amino acid antagonists to interfere with phage protein synthesis (40, 292). A brief exposure to the required amino acids or a delay in the addition of an antagonist after infection permits the continuing synthesis of phage DNA containing hydroxymethylcytosine although complete phage cannot be formed. Apparently a brief period of protein synthesis immediately after infection creates the conditions necessary for continuing phage DNA synthesis in the absence of protein synthesis. It is of interest that deoxyribonuclease activation correlates with DNA synthesis rather than continuing protein synthesis (183). The increase in ultraviolet radiation resistance with time after infection is destroyed by either type of inhibition of protein synthesis (40, 292).

Although it had seemed earlier that the synthesis of phage protein did not conform to the generalizations concerning the relation between protein synthesis and RNA synthesis, it has been established that there is an active incorporation of inorganic phosphate into the RNA of infected cells (302).

Since incorporation among the four nucleotides is heterogeneous for long periods and the total RNA does not increase, it appears that an RNA of a particular composition is synthesized. Heterogeneity of incorporation among cellular subfractions, a particulate fraction containing less than 5 per cent of the total cellular RNA being the most active (303), accords with this interpretation, which resembles results obtained in the studies of enzyme adaptation (Gale, in B). It is also of interest that synthesis of RNA and protein continue during the period of cessation of DNA synthesis when lysogenic *E. coli* and *B. megatherium* are induced by ultraviolet irradiation (142, 177).

The current view of bacteriophage infection (and, by extension, virus infections in general) involves the transmission into a host cell of the viral nucleic acids and other soluble constituents, after which the metabolism of the host becomes diverted toward the production of the infecting type of bacteriophage or virus (see Hershey in B). The chemical integrity of the injected DNA molecules after entry has been studied by Stent & Jerne (282), using T2 and T4 bacteriophages that had incorporated sufficient P^{32} to cause progressive inactivation from internal disintegrations. A distribution of the P^{32} among only 8 to 25 of the 100 to 400 descendants is calculated from the decay ("suicide") rates of the latter; the nonrandom distribution indicates that the parental DNA is not entirely degraded. Using a novel autoradiographic method that permits identification of a single DNA molecule of over 7×10^6 mol. wt. if it contains sufficient P^{32}, Levinthal (203) observes "stars" of electron tracks corresponding to 40 per cent of the DNA in shocked T2 phage being in a single piece of 45×10^6 mol. wt.; 60 per cent of the DNA-P^{32} is in molecules of less than 7×10^6 and thus not measurable. Transfer into progeny of 40 per cent of the parental phage DNA-P^{32} is observed, and half of this material forms stars when the progeny DNA is released by osmotic shock. By infection with the first progeny, phage is produced that gives stars equal in number and size to those in the first progeny, indicating that single large pieces are transferred virtually intact from one generation to the next. The commonly observed 40 to 50 per cent transfer of parental phosphorus to progeny in T2 infection stands in contrast to the 90 per cent transfer of the phosphate of phage P1 observed upon lysogenization of *Shigella dysenteriae* (133).

Tobacco mosaic virus.—The apparent identification of the functional entity of TMV with its RNA has been established independently by Fraenkel-Conrat and Schramm and their respective coworkers, though the results from the two laboratories differ in a basic operational respect. Gierer & Schramm (129, 130) have demonstrated that high-molecular-weight RNA, isolated by extraction of TMV in the presence of phenol, retains infectivity up to 4 per cent of that of intact virus. The exclusion of TMV protein or intact TMV as active contaminants in these RNA preparations has been demonstrated by their absence on serological assay, the inability of anti-TMV rabbit sera to inactivate the preparations, and the relative heat sensi-

tivity of the RNA preparations compared to intact TMV; ribonuclease, relatively inactive toward TMV, rapidly destroys the infectivity of the isolated RNA. On the other hand, Fraenkel-Conrat & Williams (117) observe that neither TMV-protein (isolated by solubilization of the virus at pH 10 to 10.5 followed by ammonium sulfate precipitation) nor TMV-RNA (isolated by detergent extraction) are capable of infectivity alone, but a mixture of the two preparations allowed to stand at pH 6.0, will reconstitute rod-like particles the microscopic appearance of which resembles intact TMV and that attain a similar small but definite fraction of the infectivity of the original virus. The reconstituted particles are somewhat more susceptible to alkali and ribonuclease than intact TMV (117, 147). The discrepancy in infectivity of the two types of the RNA preparations might be a result of the apparent extreme lability of the RNA. It is noteworthy that such preparations of Gieser & Schramm have sedimentation constants of 12 to 18S, where the preparations of Fraenkel-Conrat & Williams have constants of 8S. The former preparations, of average mol. wt. 900,000, spontaneously disaggregate to units of mol. wt. 60,000 and progressively lose infectivity (272). The view that the complete RNA aggregate is necessary for infection, which might explain the difference in behavior of these two preparations, is strengthened by the observation (189) that the breaking of a single molecular chain anywhere in the RNA of TMV by x-rays inactivates the virus particle.

That the reconstitution phenomenon itself is not a concomitant of biological specificity is indicated by the ability of polynucleotide phosphorylase polymers, when incubated with TMV-protein, to form TMV-like particles that are noninfectious (148).

Increases in the relative infectivity of reconstitued TMV, up to 10 per cent that of intact TMV, have been effected by allowing the combining process to take place slowly in 28 per cent ammonium sulfate (68, 207). Fractional elution of these reconstituted particles, as well as intact TMV, from substituted cellulose columns reveals that, with respect to the ratio of infectivity to RNA content, both are markedly heterogeneous (68).

Although it is implicit from the electron micrographic appearance of intact TMV and reconstituted TMV that the structure of the virus may consist in a simple tube-like protein shell encasing an axial core of RNA, Franklin (118), from x-ray scatter information, has concluded that the RNA is deeply imbedded in the protein.

In infected leaves, an increase in amount of insoluble nucleoprotein has been observed; as free TMV appears, this excess disappears and at the end of the infection process there is no difference in nucleoprotein content between infected and noninfected leaves (12). The increase in the four bases in this fraction is about equal to the amount that appears as TMV.

Virus composition.—That viruses include nucleic acid and protein has been appreciated since Bawden & Pirie (14) first established the presence of RNA in TMV. Most analytical work on those viruses that can be sufficiently

concentrated and purified has been directed toward determining the type and relative amount of the nucleic acid present; much less has been concerned with the detection of other constituents and making certain that these are not adventitious impurities.

From the reported RNA contents and particle weights of thirteen plant and animal viruses, Frisch-Niggemeyer (123) obtains a hyperbolic curve, which indicates an inverse relation, and shows that, regardless of source, there are about 6000 nucleotides per particle. The inverse relation between infectivity and the 8-azaguanine content of turnip yellow mosaic virus grown in the presence of 8-azaguanine is demonstrated by Matthews (224). Various strains of the RNA-containing influenza virus have been analyzed (2, 124, 125, 231), revealing RNA (0.7 to 0.9 per cent), DNA (0 to 0.1 per cent by microbiological assay) (231), and various sugars [3.5 per cent total (124), including chiefly galactose and mannose (125)]. There exist also a proportionality between infectivity and RNA content and a variation in A+U /G+C from strain A to strain B (2). Of the soluble proteins after ether disintegration, the hemagglutinin portion is a mucoprotein (4.2 per cent mannose and galactose), the antigen portion is a nucleoprotein (5.3 per cent RNA) (124).

Bacteriophage T7 has been analyzed by Lunan & Sinsheimer (215); only 71 per cent of the total phosphorus is in DNA, which shows a strict A=T, G=C correspondence, with A/G=1.08. At least two of the possible dinucleotide sequences are not found in the deoxyribonuclease digest, although the enzyme cleaves the usual 20 per cent of the phosphodiester bonds.

The glucose-containing T-even bacteriophages have been extensively analyzed to determine the relationship of glucose content to hydroxymethylcytosine content in the r and r+ varieties raised on broth and synthetic media. Although in T2 there appears to be slightly less glucose than hydroxymethylcytosine (58, 163, 279), in T4 there is a fairly exact correspondence (58, 163, 279, 301), and in T6 there is an excess of glucose (58, 163). On synthetic media, T4 and T6 show more than twice as great a glucose content as on broth, although the DNAs themselves (of T6, at least) show a closer correspondence (58).

The difficulties in freeing viruses, whether bacterial or plant or animal, from the large numbers and varieties of chemical contaminants present in their hosts, indicates at once the need for caution in interpreting analytical results. On the other hand, true components of viruses may be overlooked simply by failure to look for them in proper fashion. Cases in point are the discoveries of RNA in TMV (14) and of glucose (278, 301) and of soluble protein (153, 300) in the T-even bacteriophages. The true status of the substances found in influenza virus, just discussed, has yet to be established. However, the possible presence of hitherto undetected substances within intact viruses or within the nucleic acid preparations made from them makes it impossible at present to exclude the intervention of substances other than

nucleic acids in the biological action of viruses. Such intervention is not excluded solely by the observation that degradation of the nucleic acid component of the virus results in loss of activity.

The importance of three-dimensional structure to biological function (as with enzymes or subcellular constituents) and of trace substances to biochemical functioning (as with trace metals or enzymes) indicate possible mechanisms that may involve other substances or a role for the nucleic acids other than as macromolecules in virus infection.

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BIOCHEMISTRY OF THE STEROID HORMONES

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This review covers the period from November, 1954, to November, 1956, and considers the metabolism of steroid hormones and the action of microorganisms on steroids. The metabolism of the steroid hormones is divided into considerations of steroids isolated from various sources, the biogenesis of the steroid hormones, and their metabolic modifications as indicated by studies both *in vivo* and *in vitro*.

A number of limited and exhaustive reviews have appeared recently; these include aldosterone by Luetscher (128), Simpson & Tait (204), and Gaunt *et al.* (77A); various aspects of steroid metabolism by Samuels (191), Grant (80), Hayano *et al.* (94), Antognetti (3), Pincus (170), Levvy (121), Dorfman & Shipley (52), Dorfman (49, 50, 51), and Heard *et al.* (96); microbiological transformations by Finch (61), Florey (64), Fried *et al.* (70), Hanc & Riedl-Tumova (85), Peterson (168), Stanley & Hickey (213), Eppstein *et al.* (54), Wettstein (249), and Wettstein & Anner (250). An exhaustive and stimulating review on the mechanism of hormone action has been written by Hechter (99).

Isolation of Steroid Hormones and Related Compounds

During the past two years a variety of steroids have been isolated and identified from various natural sources (Table I). Some compounds have been isolated for the first time; others from new sources. All these new facts materially advance our knowledge of steroid metabolism.

The structures of three D-homo steroids, probably derived from 3α,17α-dihydroxypregnan-20-one by d-homoannulation, isolated from human urine have been determined by Fukushima & Gallagher (72). Four new C-20 reduced metabolites of cortisone and cortisol have been reported by Fukushima *et al.* (75). These steroids are further reduction productions of urocortisone and urocortisol, usually determined in urine as indicators of cortisone and cortisol in the body. The 3α,5β ring A reduced derivatives of corticosterone, 11-dehydrocorticosterone, and 11-desoxycortisol, in addition to corticosterone and 11-dehydrocorticosterone, have been isolated from human urine after the subjects were treated with ACTH [Dohan *et al.* (48)].

Steroids possessing oxygen functions at carbon 16 have been isolated in both the C_{18} and C_{19} series. Particularly important is the announcement by Marrian and Bauld (1955) of the isolation of 16-epiestriol, a 16β substituted steroid. This indicates that both 16α- and 16β-hydroxylations are possible, and the counterpart in the androgen series (C_{19}) is seen in the isolation and identification of androstane-3β,16β-diol, together with androstane-3β,16β-diol, from mares' pregnancy urine [Brooks & Klyne (24)]. Either or both of these two compounds could be the precursors of the

TABLE I

STEROIDS WHICH HAVE BEEN RECENTLY ISOLATED FROM TISSUES AND BODY FLUIDS

Steroid	Source	Reference
3α,17aα-Dihydroxy-17aβ-methyl-D-Homoetiocholan-17-one 3α,17aβ-Dihydroxy-17aα-methyl-D-Homoetiocholan-17-one 3α,17α-Dihydroxy-17β-methyl-D-Homoetiocholan-17a-one	Human Urine	(73)
Cortol β-Cortol Cortolone β-Cortolone	Human Urine after ACTH	(75)
Urocortisol Urocortisone Cortisol Corticosterone 3α,11β,21-Trihydroxypregnan-20-one 3α,11β,21-Trihydroxyallopregnan-3-one 11-Dehydrocorticosterone 3α,21-Dihydroxypregnane-11,20-dione 3α,17α,21-Trihydroxypregnan-20-one	Human Urine after ACTH	(48)
16-Epiestriol	Human Pregnancy Urine	(138) (244)
Androstane-3β,16α-diol Androstane-3β-16β-diol	Mares' Urine (Pregnancy)	(24)
16α-Hydroxyestrone (Previously reported as 16-Ketoestradiol-17β)	Human Pregnancy Urine	(243a) (137)
16-Ketoestradiol-17β	Human Urine (After Estradiol-17β administration)	(117)
Etiocholane-3α,16α,17β-triol Androstane-3α,16α,17β-triol Allopregnane-3β,16α,20α-triol Etiocholanediol-3α,17β-diol Allopregnane-3α,20α-diol Androstane-3α,17β-diol 3α,17β-Dihydroxyetiocholan-11-one 3α,20α-Dihydroxypregnan-11-one	Human Urine	(123)

TABLE I—(*continued*)

Steroid	Source	Reference
Estradiol-17α	Goats' Urine (Pregnancy)	(113)
Estrone Estradiol-17β Estriol	Placenta (Full term Human)	(46)
Estradiol-17β	Testicular Tumor (Human-benign)	(139)
16-Ketoestrone	Human Urine after Estrone administration	(208)
Aldosterone	Adrenal (Bovine)	(205)
Aldosterone	Adrenal (Bovine)	(87)
Aldosterone	Placenta (Human)	(134)
Aldosterone	Human Urine (Surgery Patients) Human Urine (Nephrotic Child) Human Urine (Congestive Heart Failure)	(251) (128) (130)
Androstane-3,17-dione Etiocholane-3,17-dione $\Delta^{1,4}$-Androstadiene-3,17-dione Δ^{4}-Androstene-3,17-dione	Feces (Pregnant Cow, after Progesterone Administration)	(155)
Estriol	Meconium (Human)	(112) (69)
Estrone Estradiol-17β Estriol	Semen (Human)	(45)
6β-Hydroxycortisol	Human Urine Guinea-Pig Urine	(160) (27)
6β-Hydroxytestosterone 6β-Hydroxy-Δ^{4}-Androstene-3,17-dione	Dog Liver Perfusate after Testosterone	(8)
6β-Hydroxyprogesterone	Human Placenta (Perfusion with Progesterone)	(12a)
6-Ketoprogesterone	Human Placental Perfusate with Progesterone	(84)

TABLE I—(*continued*)

Steroid	Source	Reference
2α-Hydroxycortisol	Guinea-Pig Urine	(26)
2α-Hydroxycortisol 11β-Hydroxyetiocholanolone 11β-Hydroxyandrosterone Etiocholanolone	Guinea-Pig Urine	(166)
2β-Hydroxytestosterone	Dog Liver Perfusate after Testosterone	(8)
3α,17α-Dihydroxyallopregnan-20-one Pregnane-3α,11β,20α-triol 3α,11β-Dihydroxypregnan-20-one 3α-Hydroxy-Δ^{16}-pregnen-20-one 3β-Hydroxy-Δ^{5}-Androstene-7,17-dione	Human Urine	(74)
19,21-Dihydroxy-Δ^{4}-Pregnene-3,20-dione	Adrenal (Beef)	(140)
11-Epicorticosterone (?) Cortisol Corticosterone Cortisone	Adrenal (Rat)	(53a)
Dehydroepiandrosterone Δ^{4}-Androstene-3,17-dione 11β-Hydroxy-Δ^{4}-Androstene-3,17-dione Androstane-3,17-dione (?)	Adrenal (Women with Adrenogenital Syndrome)	(17, 19)
3α(β-D-Glucuroniside)-17α,21-Dihydroxypregnane-11,20-dione	Human Urine (After Urocortisone Administration)	(198)
Δ^{4}-Androstene-3,17-dione	Human Urine (Adrenal Cancer)	(153)
Δ^{16}-Androsten-3α-ol	Human Urine (Adrenogenital Syndrome)	(153)
3α,17α,21-Trihydroxypregnan-20-one	Human Urine (Adrenogenital Syndrome with Hypertension	(22)
3α,17α,21-Trihydroxypregnan-20-one	Human Urine (Adrenal Cancer with Cushing's Disease)	(186)
3α,17α,21-Trihydroxypregnan-20-one	Human Urine (Adrenal Cancer with Cushing's Disease)	(231)
11β-Hydroxyetiocholanolone	Urine (Normal and Cancer Patients)	(172a)

TABLE I—(*continued*)

Steroid	Source	Reference
11β-Hydroxypregnanetriol 11-Ketopregnanetriol Allopregnanetriol 3α,17α-Dihydroxyallopregnan-20-one	Human Urine (Adrenal Hyperactivity)	(76)
3β-Hydroxy-$\Delta^{5(11)}$-Androsten-17-one 3β,11β-Hydroxy-Androstan-17-one	Human Urine	(111)
Urocortisone Urocortisol	Human Blood	(238)
Cortisol	Adrenal (Guinea Pigs)	(30)
Testosterone	Adrenal (Human Cancer-Adrenogenital and Cushing's Disease)	(2)
11β-Hydroxy-Δ^4-Androstene-3,17-dione	Adrenal (Hog)	(250)
Dehydroepiandrosterone Etiocholanolone 11β-Hydroxy-Δ^4-Androstene-3,17-dione	Urine (Bovine)	(101)
11β-Hydroxyandrosterone Δ^5-Androstene-3β,16α,17β-triol 3α,17α,20α-Trihydroxypregnan-11-one	Urine (Pseudohermaphrodite)	(62)

previously isolated 3β-hydroxyandrostan-16-one [Heard & McKay (98), Oppenauer (163); Huffman & Lott (105)]. Still to be isolated is the related and theoretically possible Δ^4-androstene-3,16-dione. The biosynthesis of these C_{19}-17-deoxysteroids remains to be explained.

The report of etiocholane-3α,16α,17β-triol and androstane-3α,16α,17β from human urine by Lieberman *et al.* (123) and the demonstration again of the Δ^5-3β-hydroxy compound in this series [Finkelstein *et al.* (62)] point to the likely possibility of the Δ^4-3-ketone, 16α,17β-dihydroxy-Δ^4-androsten-3-one.

In addition to epiestriol, other 16 oxygenated estrogens have been isolated, including 16-ketoestrone, 16-ketoestradiol-17β, and 16α-hydroxyestrone. Slaunwhite & Sandberg (208) isolated 16-ketoestrone after administration of estrone. This 16-ketone had previously been isolated from human urine by Serchi (200). 16-Ketoestradiol-17β was reported by Marrian (138a) but later corrected to 16α-hydroxyestrone [Marrian (137)]. However, Levitz *et al.* (117) claim that estradiol-17β is metabolized in part to the 16-keto derivative. Additional isolation of estrogens include the finding of estrone, estradiol-17β, and estriol in human semen [Diczfalusy (45)]; estriol in human

meconium [Kinsella *et al.* (112); Francis & Kinsella (69)]; the enormous quantity of 14 mg. of estradiol-17β from 180 gm. of a testicular tumor [Marti & Hesser (139)]; and estradiol-17α from the urine of pregnant goats [Klyne & Wright (113)].

Presumptive evidence for the presence of aldosterone in a variety of urines and bloods has been reported. Definitive studies include the isolation of this hormone from bovine adrenals [Simpson *et al.* (205), Harman *et al.* (87)], from human urine [Luetscher *et al.* (129, 130), Wettstein *et al.*, (251)] and from the human placenta [Majmarick & Dillon (134)]. Other studies include aldosterone-like material in calf adrenal perfusates [Ungar *et al.* (235)], human urine [Venning *et al.* (237); Axelrad *et al.* (5)], rat adrenal venous blood [Singer & Stack-Dunne (206)], and dog adrenal venous blood [Farrell *et al.* (58)].

The dried feces of pregnant cows contain androgens in reasonably high concentrations [reviewed by Dorfman & Shipley (52)] which may be increased by progesterone administration [Miller & Turner (155)]. The problem has come nearer to solution with the finding by Miller *et al.* (156) that, after progesterone treatment of the pregnant cow, the androgens androstane-3,17-dione,$\Delta^{1,4}$-androstadiene-3,17-dione, and Δ^4-androstene-3,17-dione were isolated from the feces, together with etiocholane-3,17-dione. The fact that progesterone was converted to C_{19} steroids and especially Δ^1-C_{19} steroids suggests microbiological transformation in the cow gut. As listed in Table XIV, such microbiological reactions of steroids are known.

Steroids with oxygen functions at "unusual" positions continue to be reported. The 2α-hydroxy derivative of cortisol has been isolated from guinea-pig urine [Burstein (26), Péron & Dorfman (166)]. A 2β-hydroxy steroid, 2β-hydroxytestosterone, has been isolated as a product of testosterone perfusion through a dog liver [Axelrod *et al.* (8)]. This study also uncovered the presence of a 16α-hydroxylase in dog liver. A 7-keto compound probably originating from a 7-hydroxy steroid has been isolated and identified from human urine extracts [Fukushima *et al.* (74)].

The 6β-hydroxy derivative of cortisol has been isolated from human urine [Nadel *et al.* (160)] and from guinea pig urine [Burstein *et al.* (29)]. Other 6 oxygenated compounds have been found in the progesterone series, including 6β-hydroxyprogesterone [Berliner & Salhanick (12a)] and 6-ketoprogesterone [Hagopian *et al.* (84)], and in the androgen series [Axelrod *et al.* (8)].

Adrenal tissue has yielded a 19-hydroxy derivative of deoxycorticosterone [Mattox (140)]. The 19-hydroxy substitution is of importance as a route to estrogens from the C_{19} neutral steroids [Meyer (145)], and the finding of 19-hydroxydeoxycorticosterone may indicate the presence of 19-nor and aromatic ring A C_{21} steroids in mammalian tissues.

Although indirect evidence has strongly indicated that dehydroepiandrosterone is produced by the adrenal [Dorfman & Shipley (52)] the presence of this steroid in this tissue has been established for the first time [Bloch *et al.* (17, 19)]. The question of the formation of 11α-hydroxylated steroids by adrenal tissue was raised by Eisenstein (53a) who claimed that a com-

pound in rat adrenal tissue was tentatively identified as 11-epicorticosterone. This seems unlikely since corticosterone has now been identified by infrared spectroscopy using similar experimental conditions [Koritz *et al.* (115)]. Urocortisone, the metabolite of cortisol and cortisone, has been isolated from human urine as the β-D-glucuroniside [Schneider *et al.* (198)].

Biosynthesis of Steroid Hormones

Biosynthesis of progesterone.—Progesterone has two discrete functions: first, it is a second female hormone with specific action on the uterus, vagina, mammary glands and anterior pituitary; and second, it is now known to be an important precursor of corticoids and androgens and, indirectly, of estrogens. These advances are described under the headings of biosynthesis of corticoids and androgens. In addition to these studies, progesterone biosynthesis has been demonstrated from cholesterol in the human placenta through the intermediate pregnenolone by Solomon *et al.* (210) using a perfusion technique.

Biosynthesis of corticoids.—The biosynthesis of corticoids has been discussed in detail by Roberts & Szego (179). Advances have been made concerning biosynthetic pathways to corticoid, mechanism of the hydroxylation reactions, and factors influencing the biosynthesis of aldosterone, the potent electrolyte hormone.

Pathways of corticoid biosynthesis.—The pathway cholesterol to corticoids by way of pregnenolone and progesterone has been previously established and now reinforced by recent reports (Table II). The reaction cholesterol to pregnenolone using bovine (steer) adrenal homogenate has been

TABLE II

The Biosynthesis of Corticoids

Species	Test System	Substrate	Product	Reference
Bovine	Adrenal Perfusion	Acetate	Cortisol Corticosterone	(37)
Human	*In Vivo* Adrenal Cancer	Acetate	3α,17α,21-Trihydroxy-pregnan-20-one	(38)
Guinea Pig	Adrenal (Normal and Scorbutic)	Acetate	Cortisol	(30)
Bovine	Adrenal Perfusion	Progesterone	17α-Hydroxyprogesterone 11β-Hydroxyprogesterone Corticosterone Cortisol	(118)
		17α-Hydroxy-progesterone	Cortisol	
		Pregnenolone	Progesterone Corticosterone Cortisol	

TABLE II—(*continued*)

Species	Test System	Substrate	Product	Reference
Bovine and Dog	Adrenal Homogenates	Cholesterol	Cortisol Corticosterone	(175)
Hog	Adrenal Homogenate	Acetate	Cortisol Corticosterone 11-Dehydrocorticosterone 17α-Hydroxyprogesterone Desoxycorticosterone 11-Ketoprogesterone Progesterone Δ^4-Androstene-3,17-dione	(96)
Bovine	Homogenate Supernatant (100,000 g×30 min.)	Cholesterol	Pregnenolone and Isocaproic Acid	(214)
Bovine	Adrenal Homogenate	Cholesterol	Pregnenolone Progesterone Cortisol Corticosterone	(190)
	Adrenal Mitochondrial Preparations	Cholesterol	Pregnenolone Progesterone 11β-Hydroxyprogesterone Corticosterone Cortisol	(94)
Human	*In Vivo*	Cholesterol	Urocortisol Urocortisone	(246, 247)
Rat and Hog	Adrenal Slices and Cell-free Preparations	Acetate and β,β-Dimethyl-acrylate	Corticoids Progesterone Δ^4-Androstene-3,17-dione	(4)
Bovine	Adrenal Homogenate	Cholesterol	Pregnenolone 20β-Hydroxycholesterol	(211)
Bovine	Adrenal Perfusion	Cholesterol	Progesterone Corticosterone Cortisol	(210)

confirmed and extended by Staple *et al.* (214). Human studies *in vivo* clearly demonstrated the utilization of cholesterol for corticoid biosynthesis. Doubly labeled cholesterol (both C^{14} and H^3) was administered, and the urinary metabolites of cortisone and cortisol, urocortisone and urocortisol, were isolated and were so labeled as to permit the conclusion that cholesterol was directly utilized without prior breakdown to small particles [Werbin & Le Roy (246, 247)]. A direct approach comes from Caspi *et al.* (37, 38), who examined carbons 20 and 21 of cortisol, corticosterone, and 3α,17α,21-trihydroxypregnan-20-one individually for radioactivity when biosynthesized from acetate-1-C^{14}. The latter compound was biosynthesized by a Cushing's

disease patient and was isolated from the urine. The other two corticoids were biosynthesized by the calf adrenal by a perfusion method. In each case, carbon 21 was inactive while carbon 20 was active. This distribution is what would be expected if the corticoids were derived from cholesterol, assuming that the scheme postulated by Woodward & Bloch (254) is correct. Thus these experiments are consistent with the view that the corticoids are derived from cholesterol.

A possible intermediate between cholesterol and pregnenolone has been reported [Solomon *et al.* (211)]. Incubation of cow adrenal homogenate with cholesterol-4-C^{14} resulted in the formation of 20β-hydroxycholesterol-C^{14}, but none of the following cholesterol derivatives were radioactive; 22α-hydroxy, 22β-hydroxy, 22-keto, 24β-hydroxy, and 24-keto.

Acetate-1-C^{14} and β,β-dimethylacrylate-3-C^{14} have been incubated in four rat and hog adrenal systems employing slices and cell-free preparations, and the extent of C^{14} incorporation into corticoids, progesterone, and Δ^4-androstene-3,17-dione studied. β,β-Dimethylacrylate was a far more efficient precursor of all the steroid hormones than acetate [Aprile *et al.* (4)].

Earlier reports have emphasized the possibility of a pathway of corticoid biogenesis which does not involve cholesterol as an obligatory intermediate. Stone & Hechter (217) found that ACTH increases the incorporation of cholesterol-C^{14} into corticoids by a factor of 18, while this trophic hormone only increased the incorporation of acetate by a factor of 1.4. Heard *et al.* (96), using a cell-free hog adrenal preparation with added acetate-1-C^{14}, isolated a series of corticoids all of which contained C^{14}, while the cholesterol was inactive, which is consistent with an alternative noncholesterol pathway.

Perhaps the single characteristic reaction in the biosynthesis of corticoids is that of introduction of oxygen at specific carbon atoms in progesterone. Three hydroxylations, the 11β, 17α, and 21, are of importance in the formation of corticosterone and cortisol. For the biosynthesis of aldosterone yet a fourth hydroxylation is needed, at carbon 18. Considerable progress has been made in elucidating the mechanism of these hydroxylation reactions. It has now been conclusively demonstrated that the oxygen is derived from molecular oxygen [Hayano *et al.*, (93, 94, 95); Sweat *et al.* (219)]. Hayano & Dorfman (88) observed that no deuterium entered the steroid molecule in a stable position when the 11β-hydroxylation reaction was carried out with an adrenal preparation in D_2O, nor was O^{18} present in the molecule when H_2O^{18} was employed. However, when the gas phase contained O_2^{18}, 92 per cent of the theoretical quantity of O_2^{18} was contained in the product. A similar mechanism was found for the following hydroxylations using microbiological systems, 11α, 11β, 6β, 17α, and 21.

Two recent studies strongly indicate that hydroxylation reactions require two enzymes. For 21-hydroxylation by adrenal glands, in addition to the necessary coenzymes, the 105,000 g supernatant plus washed and reprecipitated microsomes were required [Ryan (188); Ryan & Engel (189)]. Either fraction was inactive alone. The 105,000 g supernatant fraction from

the adrenal homogenate could be replaced by a similar supernatant from rat liver, but rat liver microsomes could not replace the adrenal microsomes. Tomkins (227) found similar results with the 11β-hydroxylase system. One of the two required adrenal enzymes could be replaced by a heat labile factor from rat liver.

The multiplicity of hydroxylating systems found in mammalian tissues is summarized in Table III. The following systems have been demonstrated: 2β, 6α, 6β, 11β, 16α, 17α, 18, 19, 20β (on cholesterol), and 21. The isolation of 2α-hydroxycortisol [Burstein (26); Péron & Dorfman (166)] and of a 7-ketone derivative of dehydroepiandrosterone [Fukushima *et al.* (74)] indicate the possible presence of 2α and 7α and/or 7β hydroxylating systems.

Aldosterone biosynthesis.—Desoxycorticosterone has been converted to aldosterone using a bovine adrenal homogenate [Kahnt *et al.* (109)], but, in the same system, neither progesterone nor corticosterone appeared to be converted to the salt-retaining hormone [Wettstein & Anner (250)]. In a calf adrenal perfusion system neither desoxycorticosterone nor corticosterone were efficient substrates, while the addition of progesterone to the perfusion fluid significantly increased the concentration of an aldosterone-like material [Rosemberg *et al.* (181)]. ACTH plays a minor role in the control of aldosterone production [Farrell *et al.* (57, 59); Giroud *et al.* (78); Singer & Stack-Dunne (206); Rosenfeld *et al.* (182)], while growth hormone is without

TABLE III

STEROID HYDROXYLATION REACTIONS WITH MAMMALIAN TISSUES

Position	Tissue Preparation	Substrate	Product	Reference
2β	Dog liver perfusion	Testosterone	2β-Hydroxytestosterone	(8)
6α	Bovine Adrenal Homogenate washed residue (5000×g)	Δ^4-Androstene-3,17-dione	6α-Hydroxy-Δ^4-androstene-3,17-dione	(147)
	Bovine Adrenal Homogenate washed residue (5000×g)	Δ^4-Androstene-3,17-dione	6α,11β-Dihydroxy-Δ^4-androstene-3,17-dione	(147)
6β	Adrenal perfusion	Progesterone	6β,17α-Dihydroxyprogesterone	(114)
	Bovine Adrenal Homogenate washed residue (5000×g)	Δ^4-Androstene-3,17-dione	6β-Hydroxy-Δ^4-androstene-3,17-dione	(147)
	Bovine Corpus Luteum Homogenate	Desoxycorticosterone	6β-Hydroxydesoxycorticosterone	(92)
	Rat liver perfusion	Testosterone	6β-Hydroxy-Δ^4-androstene-3,17-dione	(6)
	Rat Liver perfusion	Δ^4-Androstene-3,17-dione	6β-Hydroxy-Δ^4-androstene-3,17-dione	(6)
	Rat liver perfusion	Desoxycorticosterone	6β-Hydroxydesoxycorticosterone	(6)
	Dog liver perfusion	Testosterone	6β-Hydroxytestosterone 6β-Hydroxy-Δ^4-androstene-3,17-dione	(8) (8)
	Cirrhotic rat liver perfusion	Cortisone	6β-Hydroxydesoxycorticosterone	(6)
	Human Adrenal Slices	Progesterone 17α-Hydroxyprogesterone	Cortisol	(125)
	Rat Liver Microsomes	11-Desoxycortisol	6β-Hydroxy-11-desoxycortisol	(67) (66)

TABLE III—(*continued*)

Position	Tissue Preparation	Substrate	Product	Reference
	Human Placenta Mince	Progesterone	6β-Hydroxyprogesterone	(12a)
11β	Bovine Adrenal Homogenate washed residue (O^{18})	Desoxycorticosterone	Corticosterone-O^{18}	(93)
	Adrenal mitochondria	Progesterone	11β-Hydroxyprogesterone	(190) (25)
	Bovine adrenal mitochondria (O^{18})	Desoxycorticosterone	Corticosterone-O^{18}	(219)
	Bovine adrenal perfusion	Pregnenolone	Cortisol Corticosterone	(118)
		Progesterone	Cortisol Corticosterone 17α-Hydroxyprogesterone	
	Bovine (calf) adrenal perfusion	11-Desoxycortisol	Cortisol	(183)
		Desoxycorticosterone	Corticosterone	
		Progesterone	Cortisol	
		Dehydroepiandrosterone	11β-Hydroxy-Δ^4-Androstene-3,17-dione	
	Bovine adrenal	11-Desoxycortisol	Cortisol	(227)
16α	Dog liver perfusion	Testosterone	16α-Hydroxytestosterone	(8)
17α	Bovine Adrenal Homogenate Supernatant	Progesterone	11-Desoxycortisol	(172)
	Bovine Adrenal Homogenate Supernatant plus microsomal system	Progesterone	Desoxycorticosterone	(188) (189)
	Human Adrenal Slices	Progesterone	Cortisol	(125)
	Bovine Adrenal Perfusion	Pregnenolone	Cortisol Corticosterone	(118)
		Progesterone	Cortisol Corticosterone 17α-Hydroxy-progesterone	
	Bovine (calf) Adrenal Perfusion	Progesterone	Cortisol	(183)
18	Bovine Adrenal Homogenates	Desoxycorticosterone	18-Hydroxy-11-desoxycorticosterone	(110)
19	Bovine Adrenal Homogenate	Δ^4-Androstene-3,17-dione	19-Hydroxy-Δ^4-androstene-3,17-dione	(146)
	Bovine Adrenal Perfusion	Progesterone	19-Hydroxy-11-desoxycorticosterone 19-Hydroxy-11-desoxycortisol	(119)
	Bovine Adrenal Brei	Desoxycorticosterone	19-Hydroxy-11-desoxycorticosterone	
	Adrenal washed residue (5000×g)	Desoxycorticosterone	19-Hydroxydesoxycorticosterone	(90)
	Adrenal Homogenates	Desoxycorticosterone	19-Hydroxydesoxycorticosterone	(110)
20β	Adrenal Homogenate	Cholesterol	20β-Hydroxycholesterol	(211)
21	Bovine Adrenal Homogenate Supernatant	Progesterone	11-Desoxycortisol	(172)
	Bovine Adrenal Perfusion	Pregnenolone Progesterone	Cortisol Corticosterone	(118)
	Bovine Supernatant plus microsomes	Progesterone 17α-Hydroxyprogesterone	Desoxycorticosterone 11-Desoxycortisol	(188)
		21-Desoxycortisol	Cortisol	(189)
	Bovine (Calf) Adrenal Perfusion	Progesterone 21-Desoxycortisone	Cortisol Cortisone	(183)
9β, 11β Epoxidation	Bovine Adrenal Homogenate Washed Residue	17α,21-Dihydroxy-$\Delta^{4,9,11}$-pregnadien-3,20-dione	9β,11β-Epoxido-17α,21-dihydroxy-Δ^4-pregnene-3,20-dione (?)	(20)

effect [(78, 182); Venning (236)]. Changes in sodium and potassium concentration of body fluids produce corresponding changes in aldosterone biosynthesis or release or both [Luetscher (128); Leutscher & Axelrad (129)]; Liddle *et al.* (122) and Bartter (11) believe that the volume of extracellular fluid is more important than sodium concentrations in the regulation of aldosterone production. *In vitro* studies, however, support the idea of a direct ion effect on aldosterone production. Decreasing the sodium and increasing the potassium of a perfusion medium increased aldosterone production [Rosenfeld *et al.* (182)] and similar results were found in an *in vitro* system using the quartered rat adrenals (78).

Biosynthesis of androgens.—The understanding of the biosynthesis of androgens involves two distinct problems, one, the manner of adrenal androgen production, and the other the pathway of gonadal androgen biosynthesis. More specifically, formation of androgens by way of C_{21} steroid precursors occurs in the gonads, the adrenal cortex, and the liver. Formation of C_{19} androgens from C_{21} steroids by liver is discussed in later sections.

Table IV summarizes the newer experiments dealing with biosynthesis of androgens. Acetate is converted to androgens by the human adrenal [Bloch (17, 19)]. The most significant point is the finding of dehydroepiandrosterone, an androgen long suspected as being produced by the adrenal cortex. The appearance of androgens in human fetal adrenals before the appearance of significant amounts of corticoids [Bloch *et al.* (15, 16)] is consistent with an androgen pathway independent of the C_{21} steroids. The formation of the androgen Δ^4-androstene-3,17-dione from acetate with hog adrenal homogenate has been reported in a system which did not produce cholesterol [Bligh *et al.* (14); Heard *et al.* (96)]. These experiments shed no light on the question of direct production of androgens without the need for a C_{21} steroid intermediate.

The conversion of acetate to the androgens testosterone and Δ^4-androstene-3,17-dione is established in embrynal testicular carcinoma [Wotiz *et al.* (255)]. This study raises questions as to the intermediates and whether the reaction can occur in normal testicular tissue. Both questions have been answered by recent studies. Slaunwhite & Samuels (209) isolated the products 17α-hydroxyprogesterone, Δ^4-androstene-3,17-dione, and testosterone when rat testis slices or homogenate were incubated with progesterone. These results have been confirmed by Savard *et al.* (195), who showed similar biosynthetic reactions with rat testis and a human testicular tumor; by Lynn (132); and by Lynn & Brown (133). The latter report that in the conversion of 17α-hydroxyprogesterone to Δ^4-androstene-3,17-dione the steroid side-chain is removed as acetic acid. These newer results are consistent with earlier findings of pregnenolone in testicular tissue and thus indicate an androgen biosynthesis pathway: pregnenolone→progesterone →17α-hydroxyprogesterone→Δ^4-androstene-3,17-dione→testosterone. This scheme is applicable to androgen production by the ovary [Solomon *et al.* (212)].

TABLE IV

THE BIOSYNTHESIS OF ANDROGENS

Species	Test System	Substrate	Product	Reference
Human	Adrenal (Adrenogenital syndrome-slices)	Acetate	Δ^4-Androstene-3,17-dione Dehydroepiandrosterone 11β-Hydroxy-Δ^4-androstene-3,17-dione	(17, 19)
Rat	Testis (Particulates of Homogenate)	Progesterone 17α-Hydroxy progesterone	Δ^4-Androstene-3,17-dione plus acetic acid	(133) (132)
Human	*In Vivo*	Cholesterol (H^3+C^{14})	Androsterone Etiocholanolone 11-Ketoetiocholanolone	(246)
Rat	Testis (Homogenate and Slices)	Progesterone	17α-Hydroxyprogesterone Δ^4-Androstene-3,17-dione Testosterone	(209)
Human	Testicular Tumor	Progesterone	Δ^4-Androstene-3,17-dione Testosterone	(195)
Rat	Testis		17α-Hydroxyprogesterone	
Human	Adrenals (Hyperactive)		11β-Hydroxy-Δ^4-Androstene-3,17-dione	(40) (229)
Human	Testicular Carcinoma (Embryonal)	Acetate	Testosterone Δ^4-Androstene-3,17-dione	(255)
Human	Testicular Tumor (Interstitial Cell) Slices	Testosterone	11β-Hydroxytestosterone 11β-Hydroxy-Δ^4-androstene-3,17-dione Andrenosterone	(194, 196)
Hog	Adrenal Homogenate	Acetate	Δ^4-Androstene-3,17-dione	(14) (96)
Bovine	Ovary Homogenate	Progesterone	17α-Hydroxyprogesterone Δ^4-Androstene-3,17-dione	(212)

Biosynthesis of estrogens.—Important advances in the biosynthesis of estrogens have been made during the past two years (Table V). The overall conversion of androgens to estrogens has been clearly indicated, and this may well be the principal pathway for estrogen production. Incubation of human ovarian and stallion testicular tissue with testosterone-C^{14} resulted in the formation of estradiol-17β-C^{14}; intravenous administration of testosterone-C^{14} to a pregnant mare gave rise in the urine to estrone-C^{14}; administration of testosterone-C^{14} to an ovariectomized-adrenalectomized woman resulted in the formation of estrone-C^{14} and estradiol-17β-C^{14}; and placental, ovarian, and adrenal tissue converted Δ^4-androstene-3,17-dione-C^{14} to estrone-C^{14}. The mechanism of conversion of the C_{19} to C_{18} steroids appears to involve a 19-hydroxylated intermediate since 19-hydroxy-Δ^4-androstene-3,17-dione is relatively efficiently converted to estrone. On the other hand, this study indicated that $\Delta^{1,4}$-androstadiene-3,17-dione and 19-nor-Δ^4-

TABLE V

THE BIOSYNTHESIS OF ESTROGENS

Species	Test System	Substrate	Product	Reference
Human Bovine	Placenta (Homogenate) Ovarian Follicular Fluids	19-Hydroxy-Δ^4-androstene-3,17-dione Δ^4-Androstene-3,17-dione $\Delta^{1,4}$-Androstadiene-3,17-dione 19-Nor-Δ^4-Androstene-3,17-dione	Estrone	(145)
Dog	Ovary (Homogenate)	Acetate	Estrone Estradiol-17β	(173)
Human	Placenta Perfusion	Acetate	Estrone Estradiol-17β	(116)
Human	Ovary Tissue Slices	Testosterone	Estradiol-17β	(9, 10)
Human	*In Vivo* Adrenalectomized-ovariectomized Woman	Testosterone	Estrone	(248)
Mare	*In Vivo* Pregnancy	Testosterone	Estrone	(97)
Human	Testicular Carcinoma (Embryonal)	Acetate	Estrone Estradiol-17β	(255)
Mare	*In Vivo* Pregnancy	Acetate	Estrone Equilin Equilenin	(96)
Human	Ovary Cortical stromal hyperplasia	Testosterone	Estrone Estradiol-17β Estriol	(256)

androstene-3,17-dione were comparatively inefficient substrates, and on this basis these steroids are probably not normal intermediates in the conversion of neutral androgens to the phenolic estrogens.

On the basis of experiments *in vitro* and *in vivo* (Table V) the conversion of acetate to the estrogens estrone, estradiol-17β, equilin, and equilenin is established. One pathway of acetate to estrone and estradiol-17β is by way of C_{19} neutral steroids, as discussed above. The following evidence strongly suggests that ring B unsaturated estrogens may originate from a second pathway and perhaps that the ring B unsaturated estrogens may on reduction be converted to estrone and estradiol-17β: Estrone and the ring B unsaturated estrogens equilin and equilenin biosynthesized from acetate-C^{14} in the same experiment have significantly different specific activities [Heard *et al.* (96)]; estrone is not converted to ring B unsaturated estrogens by the pregnant mare; and testosterone is converted to estrone but not the ring B unsaturated estrogens [Heard *et al.* (97)]. These theoretical possibilities have been discussed [Dorfman (49)].

Metabolism of Steroid Hormones

Metabolism of progesterone.—The metabolism of progesterone is summarized in Table VI (*in vivo*) and Table VII (*in vitro*). Detailed studies (*in vivo*) in the human involving bile, urine, and feces and on the metabolism of

TABLE VI

The *in Vivo* Metabolism of Progesterone and Related Compounds

Species	Steroid Administered	Urinary Metabolites	Reference
Human	Progesterone	Pregnanediol (Also in Feces) Pregnanolone	(23)
Human	Progesterone	Pregnanolone Pregnanediol (Both Metabolites in Bile and Urine)	(253)
Human	Ethynyltestosterone	No Pregnanediol	(199)
Rat	Progesterone	20α-Hydroxy-Δ^4-pregnen-3-one	(252)

ethynyltestosterone have been reported. Ethynyltestosterone is not converted to pregnanediol, which implies that this steroid is not converted to progesterone.

In vitro metabolic considerations of progesterone involving hydroxylating

TABLE VII

The Metabolism of Progesterone *in Vitro* and Related Steroids

Species	Tissue Preparation	Substrate	Product	Reference
Rabbit	Liver Homogenate	Progesterone	Allopregnane-3,20-dione 3β-Hydroxyallopregnan-20-one 3α-Hydroxyallopregnan-20-one Pregnanolone	(224A)
	Liver Suspension of Disintegrated Tissue	Pregnanediol	Pregnane-3,20-dione Pregnanolone	(224)
Bovine	Corpus Luteum Homogenate	Progesterone	20β-Hydroxy-Δ^4-pregnen-3-one	(92)
Bovine	Adrenal Perfusion	Pregnane-3,20-dione	Pregnanolone Pregnane-3α,20β-diol	(174)
Rat	Liver Homogenate	Pregnenolone	Pregnenolone is metabolized to product(s) other than progesterone in air and DPN	(225)
Human	Placenta Mince	Progesterone	6β-Hydroxyprogesterone	(12A)
Human	Placenta Perfusion	Progesterone	6-Ketoprogesterone	(84)

reactions and conversion to androgens have already been discussed. Taylor has made extensive studies with progesterone and related steroids. Progesterone is reduced at positions 3, 4–5, and 20. Taylor (224A) effected the 3α, 3β, 5α and 5β reduction with a liver preparation and Hayano *et al.* (92) isolated the 20β-hydroxy derivative after incubation with bovine corpus luteum homogenate. Pregnenolone is metabolized by rat-liver homogenate in air in the presence of DPN [Taylor (225)]. Pregnanediol may be oxidized at carbons 3 and 20 [Taylor (224)] by liver tissue, and pregnane-3,20-dione may be reduced at carbon 3 and 30 by the bovine adrenal [Ralls *et al.* (174)].

Metabolism of corticoids.—The conversion of C_{21} corticoids to C_{19} androgens and related steroids is an established reaction. Studies *in vivo* continue to be reported demonstrating this metabolic point (Table VIII); androsterone and etiocholanolone from 17α-hydroxypregnenolone and 17α,21-dihydroxypregnenolone; 11-ketoetiocholanolone and 11β-hydroxyandrosterone from 21-desoxycortisol; and 11-ketoetiocholanolone and 11β-hydroxyetiocholanolone from cortisone.

The claim that 17-hydroxy-20-ketosteroids are converted to 17-desoxy-20-ketosteroids has now been withdrawn since the resulting 17-desoxy-20-ketosteroids are really artifacts (as noted in Table IX). The metabolites are actually the 17,20-dihydroxy derivatives. The artifacts resulted from the acid hydrolytic procedure causing dehydration and rearrangement. It is not possible, however, to explain the increased urinary excretion of pregnanediol after the administration of 17α,21-dihydroxypregnane-3,20-dione to a postmenopausal woman on this basis [Ungar *et al.* (230)].

TABLE VIII

THE METABOLISM OF CORTICOIDS *IN VIVO*

Species	Steroid Administered	Urinary Metabolite	Reference
Human (Addison's Disease)	17α-Hydroxypregnenolone or 17α,21-Dihydroxy-pregnenolone	Androsterone Etiocholanolone No Dehydroepiandrosterone Pregnanolone (Artifact)	(185)
	21-Desoxycortisol	11β-Hydroxyandrosterone 11-Ketoetiocholanolone 3α,17α-Dihydroxypregnane-11,20-dione	
Human (Addison's Disease)	21-Desoxycortisol	11-Ketopregnanolone (Artifact)	(184)
	17α-Hydroxyprogesterone	Pregnanolone (Artifact)	
Human	Cortisone	11-Ketoetiocholanolone 11β-Hydroxyetiocholanolone	(13)
Human	Cortisone	Cortisone Cortisol Urocortisone Urocortisol	(32)
Human	Cortisol	6β-Hydroxycortisol	(28)

TABLE VIII—(*continued*)

Species	Steroid Administered	Urinary Metabolite	Reference
Human	Cortisone	Cortisol	(41)
	Cortisone Cortisol	Urocortisone Urocortisol	
Human	Cortisol	Cortol β-Cortol Cortolone β-Cortolone	(75)
	Cortisone	Cortolone β-Cortolone (?)	
Guinea Pig	Cortisol	11β,17α,20α,21-Tetrahydroxy-Δ⁴-pregnen-3-one 11β,17α,20β,21-Tetrahydroxy-Δ⁴-pregnen-3-one 6β-Hydroxycortisol	(27)
Rat	Cortisol	11β,17α,20β,21-Tetrahydroxy-Δ⁴-pregnen-3-one (Plasma)	(1)
Human	Corticosterone	3α,21-Dihydroxypregnane-11,20-dione 3α,11β,21-Trihydroxyallopregnan-20-one 3α,11β,21-Trihydroxypregnan-20-one 3α,20α-Dihydroxypregnan-11-one	(53b)
Human	Cortisol Cortisone	Urocortisone Urocortisol	(177)
	Corticosterone 11-Dehydrocorticosterone	3α,11β,21-Trihydroxypregnan-20-one 3α,21-Dihydroxypregnane-11,20-dione	
	Desoxycorticosterone	3α,21-Dihydroxypregnan-20-one	
	11-Desoxycortisol	3α,17α,21-Trihydroxypregnan-20-one	
Human	11-Dehydrocorticosterone	3α,21-Dihydroxypregnane-11,20-dione	(79)
Rat	Cortisone	Cortisol 11β,17α,20β,21-tetrahydroxy-Δ⁴-pregnen-3-one	(34)
Human	9α-Fluorocortisol	9α-Fluorocortisol 17α,21-Dihydroxy-9α-Fluoropregnane-3,11,20-trione (?)	(245)
Human	Prednisone Prednisolone	Prednisolone Prednisone	(240)
Human	Prednisone	Prednisone (Tentative) No Cortisone, cortisol, urocortisone, or urocortisol	(82)
Human	Prednisone	Prednisone Prednisolone 11β,17α,20β,21-Tetrahydroxy-Δ¹·⁴-Pregnadien-3-one	(238)
Human	11-Desoxycortisol	3α,17α,21-Trihydroxypregnan-20-one	(186)
Human	Urocortisone	β-Cortolone Urocortisone (as β-D-glucuronoside)	(198)

TABLE IX

THE METABOLISM OF CORTICOIDS *IN VITRO*
(Hydroxylation Reactions Are Omitted but Are Included in Table IV

Species	Tissue Preparation	Substrate	Product	Reference
Guinea Pig	Liver Homogenate Microsomes	Urocortisone	Urocortisone glucuroniside	(107) (106)
Bovine	Corpus Luteum Homogenate	Desoxycorticosterone	20β,21-Dihydroxy-Δ⁴-pregnen-3-one	(92)
Bovine	Adrenal Perfusion	21-Desoxycortisone	Cortisone 17α-Hydroxyallopregnan-3,11,20-trione	(148)
Bovine	Kidney Mince	Cortisol	Adrenosterone 11β-Hydroxy-Δ⁴-androstene-3,17-dione Cortisone 11β,17α,20β,21-Tetrahydroxy-Δ⁴-pregnen-3-one	(77)
Dog	Liver Perfusion	Cortisol	(Presumptive Evidence) Cortolone Urocortisol Urocortisone 11-Epicortisol Cortisone 11β-Hydroxy-Δ⁴-androstene-3,17-dione	(7)
Rat	Liver Perfusion	Cortisone	Adrenosterone 11-Ketoandrosterone 3β,17α,20β,21-Tetrahydroxyallopregnan-11-one .1β.17α.20β,21-Tetrahydroxy-Δ⁴-pregnen-3-one 17α,20β,21-Trihydroxy-Δ⁴-pregnene-3,20-dione Cortisol 3α,17α,21-Trihydroxyallopregnane-11,20-dione 3β,17α,21-Trihydroxyallopregnane-11,20-dione 3α,11β,17α,21-Tetrahydroxyallopregnan-20-one 3β,11β,17α,21-Tetrahydroxyallopregnan-20-one	(35) (33)
Rat	Liver (Normal) Perfusion	Cortisone	Cortisol 3α,17α,21-Trihydroxyallopregnane-11,20-dione 3β,11β,17α,21-Tetrahydroxyallopregnan-20-one 3α,11β,17α,21-Tetrahydroxyallopregnan-20-one	(154)
	Liver (Cirrhotic) Perfusion	Cortisone	6β,21-Dihydroxy-Δ⁴-pregnene-3,20-dione 17α,21-Dihydroxyallopregnane-3,11,20-trione	

TABLE IX—(*continued*)

Species	Tissue Preparation	Substrate	Product	Reference
Rat	Liver Perfusion	Cortisol	17α,20β,21-Trihydroxy-Δ⁴-pregnene-3,11-dione	(33) (36)
			Allopregnane-3β,11β,17α,20β,21-pentol	
			3β,11β,17α,21-Tetrahydroxyallopregnan-20-one	
			11β-Hydroxy-Δ⁴-androstene-3,17-dione	
			3β,11β-Dihydroxyandrostan-17-one	
Rat	Liver Homogenate	11-Desoxycortisol	17α,21-Dihydroxyallopregnane-3,20-dione	(68)
			3α,17α,21-Trihydroxyallopregnan-20-one	(66)
			3β,17α,21-Trihydroxyallopregnan-20-one	(67)
			3α,17α,21-Trihydroxypregnan-20-one	
			3β,17α,21-Trihydroxypregnan-20-one	
			3β,17α,20β,21-Allopregnanetetrol	
			17α,20β,21-Trihydroxy-Δ⁴-pregnen-3-one	
			17α,20α,21-Trihydroxy-Δ⁴-pregnen-3-one	
			6β,17α,21-Trihydroxy-Δ⁴-pregnene-3,20-dione	
			Androsterone	
			Androstane-3,17-dione	
			Δ⁴-Androstene-3,17-dione	
			Etiocholanolone (tentatively identified)	
Rat	Liver Extracts	Prednisone	Urocortisone	(228)
		Cortisone	Urocortisone	
		Cortisol	Urocortisol	
		Δ¹·⁴-Androstadiene-3,17-dione	"Tetrahydro derivative"	
Rat	Liver Homogenate and Fractions	Cortisone	Cortisol	(103)
		17α,20α,21-Trihydroxy-Δ⁴-pregnene-3,11-dione	11β,17α,20α,21-Tetrahydroxy-Δ⁴-pregnen-3-one	(102)
		Urocortisone	No 11β-Derivatives	
		11-Ketoetiocholanolone		
		Cortolone and β-Cortolone		
		Urocortisol	β-Cortol	
		Urocortisone	Cortolone	
		11-Desoxycortisol	20α,21-Dihydroxy-Δ⁴-pregnen-3-one	
		Cortisol	β-Cortol	
Rat	Liver Microsomes plus Supernatant	Urocortisone	β-Cortolone	(42)
		17α-Hydroxypregnanolone	Pregnane-3α,17α,20β-triol	
			Pregnane-3α,17α,20α-triol	
Porcine	Liver Brei	Desoxycorticosterone	20α,21-Dihydroxy-Δ⁴-pregnen-3-one	(38A)
		11-Desoxycortisol	17α,20α,21-Trihydroxy-Δ⁴-pregnen-3-one	

TABLE IX

THE METABOLISM OF CORTICOIDS *IN VITRO*
(Hydroxylation Reactions Are Omitted but Are Included in Table IV

Species	Tissue Preparation	Substrate	Product	Reference
Guinea Pig	Liver Homogenate Microsomes	Urocortisone	Urocortisone glucuroniside	(107) (106)
Bovine	Corpus Luteum Homogenate	Desoxycorticosterone	20β,21-Dihydroxy-Δ^4-pregnen-3-one	(92)
Bovine	Adrenal Perfusion	21-Desoxycortisone	Cortisone 17α-Hydroxyallopregnan-3,11,20-trione	(148)
Bovine	Kidney Mince	Cortisol	Adrenosterone 11β-Hydroxy-Δ^4-androstene-3,17-dione Cortisone 11β,17α,20β,21-Tetrahydroxy-Δ^4-pregnen-3-one	(77)
Dog	Liver Perfusion	Cortisol	(Presumptive Evidence) Cortolone Urocortisol Urocortisone 11-Epicortisol Cortisone 11β-Hydroxy-Δ^4-androstene-3,17-dione	(7)
Rat	Liver Perfusion	Cortisone	Adrenosterone 11-Ketoandrosterone 3β,17α,20β,21-Tetrahydroxyallopregnan-11-one .16.17α.20β,21-Tetrahydroxy-Δ^4-pregnen-3-one 17α,20β,21-Trihydroxy-Δ^4-pregnene-3,20-dione Cortisol 3α,17α,21-Trihydroxyallopregnane-11,20-dione 3β,17α,21-Trihydroxyallopregnane-11,20-dione 3α,11β,17α,21-Tetrahydroxyallopregnan-20-one 3β,11β,17α,21-Tetrahydroxyallopregnan-20-one	(35) (33)
Rat	Liver (Normal) Perfusion	Cortisone	Cortisol 3α,17α,21-Trihydroxyallopregnane-11,20-dione 3β,11β,17α,21-Tetrahydroxyallopregnan-20-one 3α,11β,17α,21-Tetrahydroxyallopregnan-20-one	(154)
	Liver (Cirrhotic) Perfusion	Cortisone	6β,21-Dihydroxy-Δ^4-pregnene-3,20-dione 17α,21-Dihydroxyallopregnane-3,11,20-trione	

TABLE IX—(*continued*)

Species	Tissue Preparation	Substrate	Product	Reference
Rat	Liver Perfusion	Cortisol	17α,20β,21-Trihydroxy-Δ^4-pregnene-3,11-dione	(33) (36)
			Allopregnane-3β,11β,17α,20β,21-pentol	
			3β,11β,17α,21-Tetrahydroxyallopregnan-20-one	
			11β-Hydroxy-Δ^4-androstene-3,17-dione	
			3β,11β-Dihydroxyandrostan-17-one	
Rat	Liver Homogenate	11-Desoxycortisol	17α,21-Dihydroxyallopregnane-3,20-dione	(68)
			3α,17α,21-Trihydroxyallopregnan-20-one	(66)
			3β,17α,21-Trihydroxyallopregnan-20-one	(67)
			3α,17α,21-Trihydroxypregnan-20-one	
			3β,17α,21-Trihydroxypregnan-20-one	
			3β,17α,20β,21-Allopregnanetetrol	
			17α,20β,21-Trihydroxy-Δ^4-pregnen-3-one	
			17α,20α,21-Trihydroxy-Δ^4-pregnen-3-one	
			6β,17α,21-Trihydroxy-Δ^4-pregnene-3,20-dione	
			Androsterone	
			Androstane-3,17-dione	
			Δ^4-Androstene-3,17-dione	
			Etiocholanolone (tentatively identified)	
Rat	Liver Extracts	Prednisone	Urocortisone	(228)
		Cortisone	Urocortisone	
		Cortisol	Urocortisol	
		$\Delta^{1,4}$-Androstadiene-3,17-dione	"Tetrahydro derivative"	
Rat	Liver Homogenate and Fractions	Cortisone	Cortisol	(103)
		17α,20α,21-Trihydroxy-Δ^4-pregnene-3,11-dione	11β,17α,20α,21-Tetrahydroxy-Δ^4-pregnen-3-one	(102)
		Urocortisone	No 11β-Derivatives	
		11-Ketoetiocholanolone		
		Cortolone and β-Cortolone		
		Urocortisol	β-Cortol	
		Urocortisone	Cortolone	
		11-Desoxycortisol	20α,21-Dihydroxy-Δ^4-pregnen-3-one	
		Cortisol	β-Cortol	
Rat	Liver Microsomes plus Supernatant	Urocortisone	β-Cortolone	(42)
		17α-Hydroxypregnanolone	Pregnane-3α,17α,20β-triol	
			Pregnane-3α,17α,20α-triol	
Porcine	Liver Brei	Desoxycorticosterone	20α,21-Dihydroxy-Δ^4-pregnen-3-one	(38A)
		11-Desoxycortisol	17α,20α,21-Trihydroxy-Δ^4-pregnen-3-one	

Cortisol and cortisone metabolism in the human continues to attract attention. In addition to the interconversion of these two steroids, new metabolites have been reported. Burstein *et al.* (28) have reported the presence of the 6β-hydroxy derivative of cortisol and Fukushima *et al.* (75) have isolated a series of four C-20 reduced and ring A reduced derivatives of cortisol; namely, cortol, β-cortol, cortolone, and β-cortolone. β-Cortolone is a metabolite of urocortisol [Schneider (198)]. Cortisol administered to guinea pigs yielded the 6β-hydroxy derivative together with the two 20 reduced compounds 11β,17α,20α,21-tetrahydroxy-Δ^4-pregnen-3-one and 11β,17α,20β,21-tetrahydroxy-Δ^4-pregnen-3-one [Burstein & Dorfman (27)]. The latter steroid was also isolated from rat plasma after the administration of cortisol [Abelson *et al.*, (1)].

The full report of Engel *et al.* (53b) has appeared on the *in vitro* metabolism of corticosterone in a human. Four steroids have been isolated from the urine and an unusually high concentration of 5α (allo) derivatives was found. Other *in vitro* studies in this series indicate a higher concentration of the 5β (pregnane) reduced forms. Six adrenocortical steroids including cortisone, cortisol, corticosterone, 11-dehydrocorticosterone, desoxycorticosterone, and 11-desoxycortisol give rise primarily to 3α,5β tetrahydro reduced metabolites. The 11-ketone and 11β-hydroxy groups, when present, are interchangeable.

The metabolism of the newer synthetic corticoids 9α-fluorocortisol, prednisone, and prednisolone has been studied. The fluorinated steroid was excreted partly unchanged and one metabolite was tentatively identified, 17α,21-dihydroxy-9α-fluoropregnane-3,11,20-trione. Only one metabolite of the Δ^1 derivatives of cortisol and cortisone has thus far been reported. This compound is the 20β reduced derivative of prednisolone [Vermeulen (238)]. Both prednisone and prednisolone were isolated from the urine after the administration of either steroid (239). Thus far neither cortisone, cortisol, nor their ring A tetrahydro derivatives have been isolated.

On the basis of indirect evidence, the bulk of the conjugates of corticoid metabolites have been assumed to be glucuronisides. The definitive experiment of Schneider *et al.* (198) has now established that urocortisone is excreted as the β-D-glucuronoside, the complex being isolated from the urine of a subject who ingested large amounts of urocortisone.

The mechanism of glucuroniside formation of corticoids has been elucidated (Table IX, studies *in vitro*). This has been accomplished using urocortisone as substrate and a microsomal fraction from a guinea pig liver homogenate. The urocortisone is coupled with uridine diphosphate glucuronic acid (UDPGA) by the enzyme glucuronosyl transferase to yield urocortisone 3-β-D-glucuroniside identical with the complex isolated from human urine.

The perfusion of 21-desoxycortisone through a bovine adrenal resulted in the formation of cortisone by 21-hydroxylation as well as reduction of the nuclear double bond to the 5α derivative 17α-hydroxyallopregnane-3,11,20-

trione. This is in accord with the type of reductions previously described.

Cortisol incubation with a mince of bovine kidney tissue resulted in the formation of the 17-ketosteroids, adrenosterone and 11β-hydroxy-Δ^4-androstene-3,17-dione, cortisone,and11β, 17α, 20β, 21-tetrahydroxy-Δ^4-pregnen-3-one indicated that this tissuelike liver contains a side-chain splitting enzyme, an 11β-dehydrogenase and a 20β-dehydrogenase [Ganis *et al.* (77)].

Cortisol perfusion through a dog liver has indicated a variety of unique reactions on the basis of tentatively identified products [Axelrod & Miller (7)]. The finding of the 5β reduced products is contrary to the reduction products of the 5α series observed after cortisol and cortisone studies using the rat liver. A second unique finding is that of 11-epicortisol. 11α-Hydroxy compounds have only been observed as a consequence of hydroxylating reactions by microorganisms. The presence of the 11-epicorticosterone in rat adrenals has not been confirmed [Koritz *et al.* (115)].

Perfusion of cortisone through rat livers has been achieved by two groups of investigators. Caspi & Hechter (35) and Caspi (33) have isolated 10 metabolites as listed in Table IX, four of which were also reported by Miller & Axelrod (6). Of particular interest is the finding by Miller & Axelrod (6) that the cirrhotic rat liver converts cortisone to the 6β,21-dihydroxy-Δ^4-pregnene-3,20-dione implying in addition to 6β-hydroxylation, a 17-desoxylation reaction and the removal of the 11-oxygen function. These latter two reactions require further study.

Cortisol perfusion through rat livers gave rise to five products, three reduced compounds and two 17-ketosteroids [Caspi (33); Caspi & Hechter (36)].

Twelve metabolic products were isolated and identified when 11-desoxycortisol was incubated with rat liver homogenate [Forchielli (66); Forchielli & Dorfman (67); Forchielli *et al.*, (68)]. A thirteenth compound was tentatively identified. These studies are of particular importance since reduction of ring A to both 5α (allopregnane or androstane) derivatives and 5β (pregnane or etiocholane) derivatives has been demonstrated and further that the two Δ^4-5-hydrogenases have been separated. The particulate-free supernatant obtained after centrifugation at 78,000 g converted the substrate into exclusivley 5β reduced products and the particulate fractions sedimenting at 6000 g and 78,000 g reduced 11-desoxycortisol exclusively to 5α products. Still unanswered is why the 6000 g fraction, which is known to contain the Δ^4-5β-hydrogenase, yields only 5α products.

The reduction of the Δ^4 and Δ^1 nuclear double bonds has been studied in some detail by Tomkins (227), using rat liver preparations. An initial liver extract was prepared which reduced a variety of Δ^4-3-ketones including such compounds as cortisone, cortisol, and adrenosterone. When, however, this enzyme preparation was further purified by means of ammonium sulfate and calcium phosphate gel, and the fractions tested with cortisone, cortisol, and adrenosterone as substrates, distinct separation of reducing activity was evident. The data contained in Table X are consistent with the view that

TABLE X

RELATIVE ACTIVITY OF DIFFERENT RAT LIVER ENZYME PREPARATIONS WITH THREE SUBSTRATES
[Data of Tomkins (228)]

Preparation	Relative Activity		
	Cortisone	Cortisol	Adrenosterone
First Ammonium Sulfate Fraction	100	80	180
Calcium Phosphate Gel Fraction	100	60	6
Second Ammonium Sulfate Fraction	100	0	0

three different enzymes are involved. On the basis of this evidence and the work of Forchielli (66) and Forchielli & Dorfman (67), it is likely that for each Δ^4-3-ketosteroid two Δ^4-hydrogenases are present in liver tissue, one which orients the reduction of the Δ^4 group to the 5α configuration, and the other to the 5β configuration.

Rat liver contains a Δ^1-reducing enzyme in addition to the Δ^4-reducing enzymes [Tomkins (227)]. Incubation of Δ^1-dehydrocortisone and $\Delta^{1,4}$-androstadiene-3,17-dione resulted in the formation of the corresponding tetrahydro derivatives, indicating reduction of both double bonds in ring A. The Δ^1 reducing enzyme is separable from the Δ^4 reducing enzyme. In contrast with the evidence for a multiplicity of Δ^4 reducing enzymes, that is, special enzymes for each Δ^4-3-ketosteroid substrate, 3α-hydroxy dehydrogenase is nonspecific. Tomkins (227) has shown that over a wide range of enzyme purifications the relative activity toward 17α,21-dihydroxypregnane,3,11,20-trione and etiocholane-3,17-dione remained constant.

Hübener *et al.* (103) and Hübener (104) studied the reduction of the 11-keto group to the 11β-hydroxy derivative by rat liver homogenates and fractions thereof. The enzyme responsible for this transformation required the presence of a Δ^4-3-ketone group in ring A, and the substrates could be either C_{19} or C_{21} steroids.

Reduction at carbon 20 in the corticoid series was reported to be dependent on the presence or absence of a 11β-hydroxy group (103, 104). Thus cortisol and urocortisol were reduced to the 20β-form while desoxycorticosterone, 11-desoxycortisol, and urocortisone were reduced to the 20α-form. This must be limited to Hübener's specific conditions since Forchielli (66) and Forchielli & Dorfman (67) have found both 20α and 20β reduction with 11-desoxycortisol. Further, the incubation of two steroids both devoid of an 11β-hydroxy group yielded 20β-reduced products and one substrate; 17α-hydroxypregnanolone yielded both the 20α and 20β derivatives [DeCourcy & Schneider (42)].

TABLE XI

THE METABOLISM OF ANDROGENS *IN VITRO*
(For Conversion to Estrogens See Table VI)

Species	Tissue Preparation	Substrate	Product	Reference
Rat	Liver Homogenate	Testosterone	17β-Hydroxyandrostan-3-one	(187)
Rat	Liver Perfusion	Testosterone Δ^4-Androstene-3,17-dione	6β-Hydroxy-Δ^4-Androstene-3,17-dione	(6)
Rat and chick	Liver Homogenate	Δ^4-Androstene-3α,17β-diol Δ^4-Androstene-3β,17β-diol	Testosterone Δ^4-Androstene-3,17-dione Epitestosterone	(233, 234)
Rat and Bovine	Liver Homogenate and Fractions	Dehydroepiandrosterone	Dehydroepiandrosterone sulfate	(43, 44)
Rabbit	Liver Microsome-free supernatant	Testosterone Dehydroepiandrosterone Androsterones (and others including C_{21} steroids)	Corresponding sulfates	(197)
Bovine	Adrenal Homogenate	Dehydroepiandrosterone	Δ^4-Androstene-3,17-dione (Hydroxylations listed in Table IV)	(147)
Human	Serum	Testosterone	Δ^4-Androstene-3,17-dione	(178)
Rat and Human	Rat Liver Homogenate; Human Serum	Δ^4-Androstene-3,17-dione	Δ^4-Androstene-3,17-dione	(220)
Dog and Rodents	Liver Slices	Testosterone	Testosterone glucuroniside	(63)
Rat	Liver Extract (Partial Purification)	Etiocholane-3,17-dione Androstane-3,17-dione	Etiocholanolone Androsterone	(226, 228)
Dog	Liver Perfusion	Testosterone	Δ^4-Androstene-3,17-dione Epitestosterone 6β-Hydroxy-Δ^4-androstene-3,17-dione 6β-Hydroxytestosterone 16α-Hydroxytestosterone 2β-Hydroxytestosterone	(8)
Dog	Kidney Perfusion (Urine Secreted)	Testosterone	In Urine Δ^4-Androstene-3,17-dione	(124)
Rat	Testis Cell Suspensions	Δ^5-Androstene-3,17-diol	Testosterone Dehydroepiandrosterone	
		Testosterone Δ^5-Androstene-3β,17β-diol Dehydroepiandrosterone	Δ^4-Androstene-3,17-dione	(193)
		Dehydroepiandrosterone Δ^4-Androstene-3,17-dione	Trace amounts of Testosterone	

Metabolism of androgens.—Summaries of metabolic studies *in vitro* are listed in Table XI and those studies *in vivo* in Table XII.

The conversion of testosterone to 17β-hydroxyandrostan-3-one, a theoretical intermediate in testosterone metabolism, was reported for the first time [Rubin & Dorfman (187)]. 6β-Hydroxy-Δ^4-androstene-3,17-dione was isolated as a metabolite of testosterone perfusion through a rat liver [Axelrod & Miller (6)]. This finding is in keeping with other instances of 6β-hydroxylation by liver tissue.

TABLE XII

THE METABOLISM OF ANDROGENS *IN VIVO*

Species	Steroid Administered	Urinary Metabolites	Reference
Human	Epiandrosterone	Androsterone glucuronide Epiandrosterone sulfate	(108)
Guinea Pig	Testosterone	Etiocholanolone Epiandrosterone	(31)
Human	Δ^4-Androstene-3α,17β-diol Δ^4-Androstene-3β,17β-diol	Androsterone Etiocholanolone	(232)

The possibility that Δ^4-3α-hydroxy and Δ^4-3α-hydroxy steroids may be intermediate in the catabolism of testosterone has been considered [Samuels & West (192)]. These derivatives of testosterone have now been synthesized with C^{14} at C-4 and subjected to studies both *in vitro* and *in vivo*. Since both rat and chick liver preparations convert these steroids to testosterone and Δ^4-androstene-3,17-dione [Ungar *et al.* (233, 234)], their role as possible precursors of the androgens is to be considered, but they do not seem to be intermediates in the catabolic reductive pathway.

Dehydroepiandrosterone has been known to be conjugated and excreted as the sulfuric acid ester. A soluble beef and rat liver fraction containing two enzymes has been demonstrated one described as an "activating" enzyme which apparently forms an "active sulfate" intermediate and a second, a transferring enzyme which transfers the sulfate to the substrate [DeMeio *et al.* (43, 44)]. A soluble rat liver preparation formed the sulfate conjugates of androsterone testosterone, and other steroids including some in the C_{21} series as well as dehydroepiandrosterone [Schneider and Lewbart (197)].

Metabolism of estrogens.—After many years of neglect, the study of estrogens, the first steroid hormones to be isolated in crystalline form and the first steroid hormones whose structure was established, is definitely on the upswing. Newer studies (Table XIII) have contributed much to the clarification of the estrogen problem.

Beer & Gallagher (12) have made detailed and quantitative studies on the *in vivo* metabolism of estrone and estradiol-17β using C^{14} tagged material. The formation of estrone and estriol has been confirmed. The presence of estriol in species other than man has been established by Mayer (141), who recovered estrone, estradiol-17β, and estriol from the urine of the spayed dog which received estradiol-17β. Incubation of estrone with a rat liver preparations resulted in the formation of estriol [Hagopian (83)]. These studies remove the myth that estriol is an exclusive product of man. Incubation of

TABLE XIII

THE METABOLISM OF ESTROGENS *IN VIVO*

Species	Steroid Administered	Urinary Metabolite Isolated	Reference
Human	Estrone Estradiol	Estrone Estriol	(12)
Dog	Estradiol	Estrone Estradiol Estriol	(141)
Human	16-Ketoestradiol-17β	16-Ketoestradiol-17β	(117)
	Estradiol-17β	16-Ketoestradiol-17β	
Human	Estrone	16-Ketoestrone	(208)

estrone with bovine blood resulted in the formation of estradiol-17α [Levy *et al.* (120)].

The 16-oxygenated structural isomers of estrone and estradiol-17β, estrone-17 and estradiol-16 have been administered to human subjects with the demonstration of the corresponding oxidized or reduced product in the urine [Stimmell (215)]. Estrogens with oxygen at carbon 16, in addition to the oxygen function at carbon 17, have been the subjects of two new studies. Estrone-16-C^{14} has been metabolized to the 16-keto derivative [Slaunwhite & Sandberg (208)], presumably identical with the estrogen isolated from human urine by Serchi (200). The similar transformation of estradiol-17β to the 16-ketoestradiol-17β has been reported by Levitz *et al.* (117).

STEROIDS IN BLOOD

A variety of androgens, corticoids, and their metabolites have been isolated and identified in human and animal blood as detailed in Table XIV.

MICROBIOLOGICAL REACTIONS

The finding of Murray & Peterson (157) that the steroid molecule could by hydroxylated at various carbon atoms by a variety of microorganisms

TABLE XIV

STEROIDS IN BLOOD

Steroids	Source	Reference
Desoxycorticosterone	Adrenal Venous Blood (Dog)	(60)
Aldosterone	Adrenal Venous Blood (Dog)	(58)
11β-Hydroxy-Δ^4-androstene-3,17-dione Corticosterone	Adrenal Venous Blood (Rat)	(126, 127)
Androsterone	Peripheral Human Plasma	(151)
Dehydroepiandrosterone	Peripheral Human Plasma	(152)
Δ^4-Androstene-3,17-dione 11β-Hydroxy-Δ^4-androstene-3,17-dione	Human Adrenal Venous Blood	(180)
Corticosterone Cortisol 11β-Hydroxy-Δ^4-androstene-3,17-dione	Human Adrenal Venous Blood	(171)
Urocortisone Urocortisol	Human Blood	(238)

was of theoretical and practical importance. From a theoretical standpoint, it again linked microbial reactions with those known to occur in mammalian tissues and thus provided test systems for studies on mechanism. From a practical point of view, it led to mass production methods, by relatively simple means, for the highly important therapeutic agents cortisone and cortisol. The early promise of the value and importance of microbial reactions of steroids has been fulfilled in the past few years as detailed in Table XV and as reviewed by Eppstein *et al.* (54).

TABLE XV

MICROBIOLOGICAL TRANSFORMATIONS OF STEROIDS

Reaction	Substrate	Product	Microorganism	Reference
6β	19-Nortestosterone	6β-Hydroxy-19-Nortestosterone	*Rhizopus nigricans*	(167)
	Desoxycorticosterone	6β-Hydroxy-desoxycorticosterone	*Trichothecium roseum*	(150)
	Δ^4-Androstene-3,17-dione or Testosterone	6β-Hydroxy-Δ^4-androstene-3,17-dione	*Rhizopus nigricans* *Aspergillus niger*	(47)
	Desoxycorticosterone	6β-Hydroxydesoxycorticosterone	*Lensites abietina*	(249)

TABLE XV—(*continued*)

Reaction	Substrate	Product	Microorganism	Reference
	Testosterone	6β-Hydroxy-testosterone	*Rhizopus* spp.	(55)
	Progesterone	6β-Hydroxy-progesterone	*Streptomyces aureofaciens*	(65)
			Penicillium urticae	(164)
	11α-Hydroxyprogesterone	6β,11α-Dihydroxyprogesterone	*Cunninghamella blakesleeana*	(56)
	16α-Hydroxyprogesterone	6β,16α-Dihydroxyprogesterone	*Aspergillus nidulans*	(70)
	Desoxycorticosterone	6β,17α,21-Trihydroxy-Δ⁴-pregnene-3,20-dione	*Cephalothecium roseum*	(144)
	Desoxycorticosterone	6β-Hydroxydesoxycorticosterone	*Rhizopus arrhizus*	(95)
7α	Progesterone	7α-Hydroxy-progesterone	*Physomyces blakesleeanus*	(70)
	Desoxycorticosterone	7α-Hydroxy-desoxycorticosterone	Curvulariae: *Pezizа* sp.	(150)
7C=0	Pregnenolone	3β,11α-Dihydroxy-Δ⁵-pregnene-7,20-dione	*Rhizopus arrhizus*	(143)
	3β-Hydroxyallopregnan-20-one	3β,7β-Dihydroxyallopregnan-20-one	*Rhizopus arrhizus*	(143, 158)
	Pregnenolone	3β, 7β-Dihydroxy-Δ⁵-pregnen-20-one	*Rhizopus nigricans*	(143)
8ξ or 9ξ	Progesterone	8ξ or 9ξ-Hydroxyprogesterone	*Streptomyces aureofaciens*	(70)
	Desoxycorticosterone	8-Hydroxy-desoxycorticosterone	Mucor spp.; *helicostylum piriforms*	(143) (142, 159)
		8β- or 9α-Hydroxydesoxycorticosterone	*Neurospora crassa*	(216)
	11-Desoxycortisol	8ξ,17α,21-Trihydroxy-Δ⁴-pregnene-3,20-dione	Mucor spp.; *Helicostylum piriforme*	(157) (142)
	Desoxycorticosterone	8β?-Hydroxy-desoxycorticosterone	*Curvularia pallescens*	(249)
10ξ	19-Nortestosterone	10ξ-Hydroxy-19-Nortestosterone	*Rhizopus nigricans*	(167)
11α	Desoxycorticosterone	Epicorticosterone	*Rhizopus nigricans*	(95)
	19-Nortestosterone	11α-Hydroxy-19-Nortestosterone	*Rhizopus nigricans*	(167)
	Δ⁴-Androstene-3,17-dione	11α-Hydroxy-Δ⁴-Androstene-3,17-dione	*Rhizopus nigricans*	(55)
	Testosterone	11α-Hydroxytestosterone	Rhizopi	(55)
	Methyltestosterone	11α-Hydroxy-methyl-testosterone	Rhizopi	(55)
	16,17-Oxidoprogesterone	11α-Hydroxy-16,17-Oxidoprogesterone	Rhizopi	(169)
	Progesterone	11α-Hydroxyprogesterone	*Pestalotia foedans*	(203)
	Desoxycorticosterone	11α-Hydroxydesoxycorticosterone	Aspergilli	(56)
	16α-Hydroxyprogesterone	11α,16α-Dihydroxyprogesterone	*Aspergillus niger*	(70)
	Progesterone	11α,17α-Dihydroxyprogesterone	*Cephalothecium roseum*	(144)
	Desoxycorticosterone	Epicortisol	*Cephalothecium roseum*	(144)
	11-Desoxycortisol	Epicortisol	*Aspergillus nidulans*	(47)
			Helicostylum piriforme	(142)
	3β-Hydroxyallopregnan-20-one	3β,11α-Dihydroxyallopregnan-20-one	*Rhizopus nigricans*	(143)
	Progesterone	11α-Hydroxyprogesterone	*Aspergillus ochraceus*	(53)

TABLE XV—(*continued*)

Reaction	Substrate	Product	Microorganism	Reference
11β	Progesterone	11β-Hydroxyprogesterone	*Curvalariae*	(201)
			Cunninghamella blakesleeana	(86)
	17α-Hydroxyprogesterone	21-Desoxycortisol	*Curvularai lunata*	(202)
		11β,14α,17α-Trihydroxy-Δ⁴-pregnene-3,20-dione		(201)
	Desoxycorticosterone	Corticosterone		
	11-Desoxycortisol	Cortisol		
	11-Desoxycortisol	Cortisol	*Cunninghamella blakesleeana*	(162)
		Cortisone		(135, 136)
14α	Δ⁴-Androstene-3,17-dione	14α-Hydroxy-Δ⁴-Androstene-3,17-dione	*Helicostylum piriforme*	(142)
	Testosterone	14α-Hydroxytestosterone		
	Progesterone	14α-Hydroxyprogesterone	*Bacillus cereus*	(70)
			Helicostylum piriforme	(142)
	Desoxycorticosterone	14α-Hydroxydesoxycorticosterone	*Cunninghamella blakesleeana*	(135)
	Desoxycorticosterone	14α-Hydroxydesoxycorticosterone	*Helicostylum piriforme*	(159)
			Cunninghamella blakesleeana	(136)
15α	Δ⁴-Androstene-3,17-dione and Testosterone	15α-Hydroxy-Δ⁴-Androstene-3,17-dione	*Fusarium* sp.	(143)
	Testosterone	15α-Hydroxytestosterone	*Fusarium* sp.	
	Progesterone	15α-Hydroxyprogesterone	*Colletotrichum antirrhini*	(70)
			Gibberellae	(143)
			Fusaria	
			Penicillum urticae	(164)
			Phycomyces blakesleeanus	(70)
	Desoxycorticosterone	15α-Hydroxydesoxycorticosterone	Gibberellae	(143)
				(150)
			Fusaria	(143)
15β	Desoxycorticosterone	15β-Desoxycorticosterone	*Lenzites abietina*	(143)
				(47)
				(150)
16α	Δ⁴-Androstene-3,17-dione	16α-Hydroxy-Δ⁴-Androstene-3,17-dione	*Streptomyces roseochromogenus*	(70)
	Desoxycorticosterone	16α-Hydroxydesoxycorticosterone	*Streptomyces* sp.	(70)
				(243)
			Streptomyces roseochromogenus	(70)
			Didymella rodakii	(249)
	Progesterone	16α-Hydroxypregnane-3,20-dione	*Streptomyces* sp.	(165)
17α	Desoxycorticosterone	11-Desoxycortisol	*Cephalothecium roseum*	(168)
			Trichothecium roseum	(149)
	Corticosterone	Cortisol	*Cephalothecium roseum*	(144)
			Trichothecium roseum	(149)
	Desoxycorticosterone	6β,17α,21-Trihydroxy-Δ⁴-pregnene-3,20-dione	*Cephalothecium roseum*	(144)
		11α,17α,21-Trihydroxy-Δ⁴-pregnene-3,20-dione		
	Progesterone	17α-Hydroxyprogesterone	*Cephalocethium roseum*	(95)
	Progesterone	Desoxycorticosterone	*Ophiobolus herbotrichus*	(149)
	11-Ketoprogesterone	11-Dehydrocorticosterone	*Aspergillus niger*	(257)

TABLE XV—(*continued*)

Reaction	Substrate	Product	Microorganism	Reference
	11β-Hydroxyprogesterone	Corticosterone	*Aspergillus niger*	(257)
	17α-Hydroxyprogesterone	11-Desoxycortisol	*Ophiobolus herbotrichus*	(149)
	19-Norprogesterone	19-Nordesoxycorticosterone		
	6β-Hydroxyprogesterone	6β-Hydroxydesoxycorticosterone	*Aspergillus niger*	(257)
	Progesterone	Desoxycorticosterone	*Cephalocethium roseum*	(95)
17β-OH⇌17-C=O	Estrone	Estradiol-17β	*Pseudomonas* sp.	(221)
	Estradiol-17β	Estrone		
	Testosterone	6β-Hydroxytestosterone	*Fusarium* sp.	(143)
	Adrenosterone	11-Ketotestosterone	Yeast	(100)
3α-OH⇌3-C=O	Androsterone	Androstane-3,17-dione	*Pseudomonas* sp.	(222)
3β-OH⇌3-C=O	Δ⁵-Androstene-3β,17β-diol	Testosterone	*Corynebacterium mediolanum*	(39)
20β-OH⇌20-C=O	16α-Hydroxyprogesterone	16α,20β-Dihydroxy-Δ⁴-pregnen-3-one 16α,20β-Dihydroxy-Δ¹·⁴-pregnadien-3-one	*Streptomyces lavendulae*	(70)
	Progesterone	20β-Hydroxy-Δ⁴-pregnen-3-one		
	Desoxycorticosterone	20β,21-Dihydroxy-Δ⁴-pregnen-3-one	*Streptomyces* sp.	(54)
$C_{21} \rightarrow C_{19}$	Progesterone	Δ¹-Dehydrotestololactone	*Fusarium caucasicum*	(56)
	Progesterone	Δ¹·⁴-Androstadiene-3,17-dione Δ¹-Dehydrotestololactone	*Fusarium solani*	(161)
	Progesterone	Δ¹-Dehydrotestololactone	*Fusarium caucasicum*	(56)
Formation of Δ¹ Group	Cortisone	Prednisone	*Fusarium solani*	(241)
			Corynebacterium simplex	(161a)
	9α-Fluorocortisol	9α-Fluoroprednisolone	*Corynebacterium simplex*	(161a)
	Progesterone	Δ¹-Dehydroprogesterone	*Calonectria decora*	(241)
	Desoxycorticosterone	Δ¹-Dehydrodesoxycorticosterone	*Calonectria decora*	(241)
			Corynebacterium simplex	(161a)
	Corticosterone	Δ¹-Dehydrocorticosterone		
	Cortisol	Prednisolone	*Corynebacterium simplex*	(161a)
	Methyltestosterone	Δ¹-Dehydromethyltestosterone	*Didymella*	(242)
	17α-Ethinyltestosterone	Δ¹-Dehydro-17α-Ethinyltestosterone		
	Δ¹¹-Dehydroprogesterone	Δ¹·⁴·¹¹-Pregnatriene-3,20-dione		
	Progesterone	Δ¹-Dehydroprogesterone	*Fusarium solani*	(161)
	Cortisone	Prednisone	*Bacillus sphaericus*	(218)
	Cortisol	Prednisolone		
	9α-Fluorocortisol	9α-Fluoroprednisolone		
	Corticosterone	Δ¹-Dehydrocorticosterone		
	Desoxycorticosterone	Δ¹-Dehydrodesoxycorticosterone		
	11-Desoxycortisol	Δ¹-Dehydro-11-desoxycortisol		
	Progesterone	Δ¹-Dehydroprogesterone		
	Δ⁴-Androstene-3,17-dione	Δ¹·⁴-Androstadiene-3,17-dione		
	11β-Hydroxyprogesterone	Δ¹-Dehydro-11β-hydroxyprogesterone		
	11-Dehydrocorticosterone	Δ¹-Dehydro-11-dehydrocorticosterone		
Δ⁵-3β-OH→Δ⁴-3-C=O	Δ⁵-Androstene-3β,17β-diol	Testosterone	*Corynbacterium mediolanum*	(39)

TABLE XV—(*continued*)

Reaction	Substate	Product	Microorgansim	Reference
$\Delta^{9(11)}\rightarrow$ 9β,11β-Epoxide	17α,21-Dihydroxy-$\Delta^{4,9(11)}$-Pregnadiene-3,20-dione	17α,21-Dihydroxy-Δ^{4}-9β,11β-epoxidopregnene-3,20-dione	*Curvalaria lunata* *Cunninghamella blakesleeana*	(21)
$\Delta^{14}\rightarrow$14α,15α-Epoxide	17α,21-Dihydroxy-$\Delta^{4,14}$-pregnadiene-3,20-dione	17α,21-Dihydroxy-Δ^{4}-14α,15α-epoxidopregnene-3,20-dione	*Curvalaria lunata* *Cunninghamella blakesleeana* *Helicostylum piriforme* *Mucor griseocyanus* *Mucor parasiticus*	(21)
Δ^{4}-3-C=O→ Δ^{1}-3-C=O	Δ^{4}-Androstene-3,17-dione	Δ^{1}-Androstene-3,17-dione	*Pseudomonas**	(223) (220)
$\Delta^{5(10)}$-3-C=O→Δ^{1}-3-C=O	17β-Hydroxy-$\Delta^{5(10)}$-estren-3-one	19-Nortestosterone	*Pseudomonas**	(223)

* Grown on testosterone.

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HAEM PIGMENTS AND PORPHYRINS[1,2]

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Introduction

The pyrrole pigments were reviewed by Lemberg & Legge in 1950 (1) just after glycine had been identified as a specific precursor of haem in duck erythrocytes but before the origin of the majority of the carbon atoms in the porphyrin ring had been definitely elucidated. In the intervening six years the subject has developed enormously and widespread interest in the chemistry and biochemistry of the porphyrins has been aroused. In particular, the biosynthesis of the prosthetic groups of haemoglobin and chlorophyll have been studied in different organisms and many reviews have appeared summarizing aspects of the work. The responsibility of a review article such as this is to place developments in historical perspective. The writer will therefore describe the story from 1950 up to the beginning of this year in general, and deal in more detail with work performed in 1956. The chemistry of cytochrome prosthetic groups, which is still developing, will receive only incidental mention. Kinetic studies of haem catalysis will be left for a future review.

Reviews

Books on porphyrins have appeared by Brugsch (2) in 1952 and by Vannotti (3) in 1954 while the Ciba Foundation Symposium volume on *Porphyrin Biosynthesis and Metabolism* (4) in 1955 presents an authoritative account up to February 1955 of this most rapidly growing aspect of our subject. Chemistry and biology of the bile pigments are presented in monographs by Gray (5) in 1953 and With (6) in 1954, and an important review by Lemberg (6a) of bile pigment formation appeared in 1956. Porphyrin metabolism has been covered by several authors, including Muir (7), Watson (8) and Rimington (9) in 1952, Bénard, Gajdos & Gajdos-Török (10) in 1953 and Siedel (11), Weatherall (12) and Lemberg (13) in 1954. A symposium on the chemical pathology of animal pigments (14) was also held in 1954. Reviews in 1955 include Orten (15), Lemberg (16) and Rimington (17, 18). Mention must also be made of Linstead's (19) Pedler Lecture in 1953 and Granick's (20) Harvey Lectures of 1950 which cover specialized topics.

The biosynthesis of porphyrins and haem has received special attention in reviews by Falk & Rimington (21) in 1950, Zeile (22) in 1954, Popják (23) in 1955, Rimington (18, 24, 25) in 1955 and 1956, and Shemin (26), (cf. also

[1] The survey of the literature pertaining to this review was completed in October, 1956.

[2] The following abbreviation is used in this chapter: APAC for allylisopropylacetylcarbamide.

4). Four other general surveys may be mentioned: Klüver (27) on porphyrins in relation to the development of the nervous system; Nizet (28) on erythropoiesis and protein metabolism; Klotz & Klotz (29) on the structure of the oxygen-carrying proteins; and Aldrich, Labbe & Talman (30) on porphyrin metabolism with special reference to childhood.

Porphyrin and Pyrrole Chemistry

To the chemist, confirmation of Fischer's postulated structure for uroporphyrin I was badly needed. MacDonald & Stedman (31) have supplied proof of its correctness by analytical work and finally by syntheses; the octamethyl ester had m.p. 290–292°. Synthesis of uroporphyrins II and IV have also been achieved by MacDonald and Michl (4). The uroporphyrin III synthesized by Treibs & Ott (32) is found by MacDonald (33) to be impure, containing the II isomer as did the synthetic mixture of uroporphyrins first obtained by MacDonald (34) in 1952. It is only a question of time before pure synthetic uroporphyrin III will also be available.

The characterization of these isomers by means of infrared spectroscopy, x-ray diffraction, etc., is of the greatest importance to the worker with biological systems who has usually to separate complex mixtures and identify the constituents. Uroporphyrins I and III can be separated completely by paper chromatography (35, 36) and the four coproporphyrins into three spots, isomers I, II, and III plus IV respectively (4). No micromethod is yet available which separates coproporphyrins III and IV or uroporphyrins III and IV; it is badly needed.

Porphine, the prototype of the porphyrin series, was synthesized in minute yield by Fischer & Gleim (37) and by Rothemund (38) in 1936, but judging from reports of the spectral studies on these materials, the purity of the latter must be highly suspect. A method of synthesizing chlorin in yields of over 3% and the oxidation of this to porphine has been reported by Eisner & Linstead (39, 40); chlorin is shown to be dihydroporphine. The technique used by these workers of dehydrogenation by tetrachloro-1:2-benzoquinone has been applied by Whalley (41) to a quantitative study of some azaporphines. Linstead *et al.* (42) argue from the spectroscopic similarity of chlorin and methylpyrophaeophorbide that the two are at the same level of hydrogenation, namely the dihydroporphine level, as first suggested by Stoll & Wiedemann (43). By degradation of the pyrophaeophorbide and isolation of an optically active dihydrohaematinic imide they fix the location of the two "extra" hydrogen atoms at the β positions of ring IV. They also show grounds for assigning a *trans* configuration to the natural chlorophylls.

Four more deuteroporphyrins have been synthesized by Warburg, Gewitz & Völker (44) and by controlled oxidation of protoporphyrin, Lemberg & Parker (45) have obtained and separated chlorocruoroporphyrin (2-formyl-4-vinyl-deuteroporphyrin IX) and diformyldeuteroporphyrin IX.

Visible absorption spectra.—Linear combinations of atomic orbital-molecular orbital calculations were applied to porphine and tetrahydropor-

phine in 1950 (46 to 49); Matlow (50), using this combination, finds two possible forms of porphine (those with hydrogens adjacently and oppositely bonded to the N atoms of the pyrrole nuclei) to have identical ground energy states. The suggestion is made that the peaks in the spectrum of porphine are not due mainly to vibrational fine structure of a single electronic transition but rather are the combination of the electronic spectra of the two isomers mentioned above. The electronic structure and excitation of porphyrins are dealt with in a masterly chapter by Platt (51). Concerning porphine itself, the present reviewer feels that accurate spectral data derived from absolutely pure material is required upon which to base conclusions; previously measured samples have certainly been impure and reliable data on porphine obtained by a new synthesis are being accumulated in his laboratory. LCAO-molecular orbital calculations by Barnard & Jackman (52) indicate that in bacteriochlorophyll, which is at the tetrahydroporphyrin level, the two pairs of H atoms are located on opposite pyrrole rings. The polarization spectra of chlorophyll derivatives and their bearing on molecular structure are discussed by Goedheer (53).

Infrared absorption spectra.—Infrared absorption has been used to compare porphyrin samples of different origin. The position isomers, coproporphyrins I and III were shown by Gray, Neuberger & Sneath (54) to exhibit spectral differences. The first systematic infrared examination of 35 different porphyrins and haems by Falk & Willis (55) has not only provided reference spectra but also permits some general conclusions regarding frequencies due to functional groups and the influence thereon of substituents in the porphyrin molecule. Craven, Reissman & Chinn (56) state that the infrared spectra of coproporphyrins I and III cannot be differentiated, which is contrary to work already quoted (54, 55). In all such studies chemical characterization and evidence of purity is absolutely essential.

X-ray diffraction.—Kennard & Rimington (57) have published data for the methyl esters of coproporphyrins I and III, uroporphyrins I and III, mesoporphyrins I and IX, protoporphyrin IX and deuteroporphyrin IX. The diffraction patterns of coproporphyrins II and IV (from Fischer's collection) are reported by Watson & Berg (58) as well as those of the I and III isomers and some uroporphyrins; Watson, Berg & Hawkinson (59) report that of the heptacarboxylic porphyrin of m.p. 208°. These papers draw attention to the difficulties of interpretation.

Structure of Vitamin B_{12}.—An outstanding achievement to the credit of British biochemistry has been the elucidation of the structure of cyanocobalamin and the recognition of a series of "vitamins B_{12}" in which different groups are attached to the cobalt atom. The structure was announced by a team of workers headed by Hodgkin *et al.* (60) and followed closely a symposium organized by the Biochemical Society (61). The cobalamins are of interest in porphyrin chemistry on account of their ring system. This contains four pyrrole groups, two of which are united directly and the remainder through bridge carbon atoms. In this connection, the synthesis of a highly

colored compound with two directly linked, partially reduced pyrrole rings by Elvidge, Fitt & Linstead (62) deserves attention.

Porphobilinogen.—Waldenström (63) gave the name "porphobilinogen" to the substance present in acute porphyria urines, which gives a red dye, insoluble in chloroform, with Ehrlich's p-dimethylaminobenzaldehyde reagent (64). Its properties were studied in concentrates by Waldenström & Vahlquist (65), Prunty (66), Grieg, Askevold & Sveinsson (67), Formijne & Poulie (68) and others, but isolation was first accomplished by Westall (69). The structure of porphobilinogen was elucidated by Cookson & Rimington (70) and was supported by Cookson (71), Kennard (72), Granick & Bogorad (73) and enzymic experiments to be noticed later. It is a substituted pyrrole

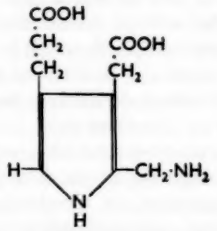


FIG. 1. Porphobilinogen.

(Fig. 1) which has played a decisive part in revealing the biosynthetic pathway to haem. It is easily transformed chemically into a mixture of uroporphyrins (65, 69, 70). Carboxyporphobilinogen, synthesized by Prasad & Raper (74), was partially decarboxylated by Rimington & Krol (75) by warming with copper acetate in pyridine to yield chromatographically identified porphobilinogen. Jackson, MacDonald & MacDonald (76) have obtained porphobilinogen identical in all respects with natural material by way of carboxyporphobilinogen which was first lactamized and the α carboxyl group then eliminated by heating with water, the lactam ring being reopened to give porphobilinogen. Scott (77) has described conditions under which porphobilinogen can be obtained chemically from δ-aminolaevulic acid.

Pyrroles.—Attention may be drawn to the survey by Cookson (78) of the ultraviolet spectra of some 25 representative pyrrole derivatives.

ANALYTICAL METHODS

The determination of urinary coproporphyrin has been studied by Schwartz, Zieve & Watson (79) who introduce an improved technique, including treatment with dilute iodine to oxidize any porphyrinogen to porphyrin. Much discussion was current concerning precursors of porphyrins present in urines; Schwartz *et al.* showed that as much as 50 per cent of the total urinary coproporphyrin could be present as coproporphyrinogen. Revised figures for normals are given by Zieve *et al.* (80).

The sharp absorption bands with high specific extinctions, which all porphyrins display, makes them ideally suited to spectrophotometric determination once the molecular extinction coefficients are known and absorbing impurities are removed or can be allowed for. A generally applicable correction formula for use with impure solutions is given by Rimington & Sveinsson (81), who also report specific extinction coefficients for uroporphyrins I and III. Their constants and those for the coproporphyrins and protoporphyrin are summarized by With (82). Measurements on coproporphyrin are also given by Brugsch & Kubowitz (83) and on haematoporphyrin by Legrand (84). The determination of uroporphyrin has always presented difficulties as it is insoluble in the usual organic solvents. A direct extraction method employing cyclohexanone, suitable for fluids such as urine, is now described by Dresel, Rimington & Tooth (85). With (86) extracts uroporphyrin from bones, teeth, and shells with 0.5N HCl and applies spectrophotometry with use of the correction formula for quantitative determination. Eriksen (87) has proposed improvements in the earlier method for quantitative urinary porphyrin analysis of Sveinsson, Rimington & Barnes (88).

Partition chromatography.—This is well reviewed up to 1954 by Falk (89). Paper chromatography, was first applied to porphyrins for separation and identification by Nicholas & Rimington (90). Eriksen (91) substituted the 2,6 isomer for the original mixture of lutidines and his method has now been developed into a very sensitive technique by Falk *et al.* (92; see also 4 and 18) whereby, for example, three of the four coproporphyrin isomers may be separated from each other. Coproporphyrins III and IV have the same R_F.

Paper chromatography of extracts from biological materials has, on numerous occasions, revealed the presence in such mixtures of porphyrins presumed to bear eight to one carboxylic function per molecule. Some of these pigments have been isolated as crystals by Macgregor, Nicholas & Rimington (93).

Paper chromatographic separation of porphyrin methyl esters was described by Chu, Green & Chu (94) but uroporphyrins I and III failed to separate in their system. Falk & Benson (36) achieved this separation using a kerosene:dioxan mixture and by combination with Chu, Green & Chu's method they could separate completely a mixture of uroporphyrins I and III, coproporphyrins I and III, and protoporphyrin.

The lutidine methods of paper chromatography applied to unesterified uroporphyrin samples almost invariably give a "double spot." A component running slightly faster than uroporphyrin III ester is also visible on dioxan chromatography. Careful column chromatography ($CaCO_3$) by Falk *et al.* (92) has achieved a separation of these components. The faster running is named "pseudouroporphyrin" but its nature is not yet clear. It cannot be one of the four known position isomers of uroporphyrin since isomers I and II run together and also isomers III and IV in the dioxan method. The be-

havior of the heptacarboxylic porphyrin (with ester m.p. 208°) of Watson, Schwartz & Hawkinson (95) is quite different. There is a close similarity, however, between pseudouroporphyrin and the uro-like porphyrin (with ester m.p. 211–216°) reported by Canivet & Rimington (96). The latter yielded coproporphyrin III on decarboxylation.

The iron complexes of porphyrins are separable by the lutidine system of Nicholas & Rimington (90). Chu & Chu (97) have introduced a reversed phase technique using a water—*n*-propanol—pyridine system for the free haemins or kerosene—chloroform—*n*-propanol for their methyl esters. Chu & Chu (98) also describe several systems for the separation of the dicarboxylic porphyrins. Kehl & Stich (99) have applied paper chromatography to the separation of some bile pigments.

Column chromatography.—Falk (89) has summarized the developments up to 1954. Great difficulty still exists in defining the adsorption characteristics of any batch of adsorbent and reproducibility of results by other workers is often impossible. Watson, Berg & Hawkinson (100) have endeavored to standardize calcium carbonate by means of an "iodine number."

Countercurrent distribution.—Papers by Paul (101) and Granick & Bogorad (102) describe application of the Craig technique to the separation and purification of porphyrins. The pigments behave as ideal solutes in suitably chosen two-phase systems. The technique is extremely sensitive, revealing even traces of impurity, and can naturally be used as a preparative procedure and for establishment of identity with reference materials on a micro scale. Falk *et al.* (92) have used the method to study the stability of protoporphyrin in different strengths of hydrochloric acid. Analysis of porphyrin mixtures is described by Torres & de la Gandara (103).

Electrophoresis.—The porphyrins are ampholytes. Their ionophoretic separation was first described by Papastamatis & Kench (104) who used agar gel as the supporting medium and a phosphate buffer of pH 8. Electrophoresis on paper offers several advantages and has been successfully used by Heikel [(105), who added a correction to With's paper (82).] Heikel used barbital, buffered at pH 8.6. The method has also been used by Larsen, Melcer & Orten (106) with borate buffered at pH 11, and by With (107). None of these methods effected separation of the I and III isomers of coproporphyrin or uroporphyrin. Heikel (108) obtained a pH-mobility curve for porphobilinogen by paper electrophoresis similar to that found by Waldenström & Vahlquist (65) using the Tiselius apparatus. The technique may prove very useful for purification purposes and for identifying porphobilinogen in specimens of urine, etc.

Special methods of determination.—Complex formation between a porphyrin and copper is accompanied by a great change in absorption spectrum. Upon this fact Oliver & Rawlinson (109) have based an ingenious method for quantitative determination of small quantities of any porphyrin without isolation and even in the presence of impurities. The technique involves titra-

tion of porphyrin with standard copper solution, heating to form the complex, and following the change in optical density at selected wavelengths where the porphyrin and its copper complex show greatest differences in absorption.

Microbiological methods for the assay of substances required by the test organisms for growth or acid production are now extensively used. Lascelles (110) has, however, described a rather more unusual assay, that of iron protoporphyrin using a variant strain of *Staphylococcus aureus*. The organism grows aerobically in the presence of haemin but not of protoporphyrin plus iron. Haemin is rendered nonessential if the medium is supplemented with acetate or pyruvate and such cells, so grown and then incubated in buffered glucose, are capable of reducing nitrate to nitrite only if haemin is also present; the extent of reduction is, moreover, proportional to the quantity of haemin added in the mixture to be assayed. A most interesting paper (in Polish with English summary) is contributed by Pawelkiewicz & Zodrow (111) on the formation of coproporphyrin III and traces of the I isomer together with δ-aminolaevulic acid by *Propionibacterium shermanii*. A selective method for the detection of δ-aminolaevulic acid in bacterial cultures is described and a spectrophotometric method for determining porphyrins (based on 81). Raising the Fe content of the medium lowers the porphyrin production but raises that of δ-aminolaevulic acid. Added cobalt, which inhibits porphyrin production, is found largely in the form of cobalamin components.

Determination of porphobilinogen and δ-aminolaevulic acid.—Vahlquist's (112) method for determination of porphobilinogen in urine is not very accurate; moreover the color reaction with Ehrlich's p-dimethylaminobenzaldehyde reagent may be inhibited or completely suppressed in some urines [Goldberg & Rimington (113)]. Marked differences in porphyrin formation on heating urines are also apparent, leading Watson (114) to suggest the occurrence of two forms of porphobilinogen. Formijne & Poulie (4, 115) have studied the Ehrlich reaction and propose an improved method dependent upon a spectrophotometric correction formula. Both Mauzerall & Granick (116) and Rimington, Krol & Tooth (117) base quantitative methods upon preliminary separation of porphobilinogen in urine, using ion-exchange resins and then color development with a modified Ehrlich reagent. Both discuss the mechanism of the reaction in the light of work by Treibs & Herrmann (118); the former also describe determination of δ-aminolaevulic acid after concentration from urine upon an ion-exchange resin and application of the method of Granick & Vanden Schrieck (119). They find small quantities of both porphobilinogen and δ-aminolaevulic acid in normal urine. Rimington *et al.* (117) show that compounds such as cysteine, thiourea and thiouracil profoundly affect porphyrin production from porphobilinogen by heating but the effects depend not only on pH but also on whether the solvent is water or urine.

Two methods for determination of δ-aminolaevulic acid are described

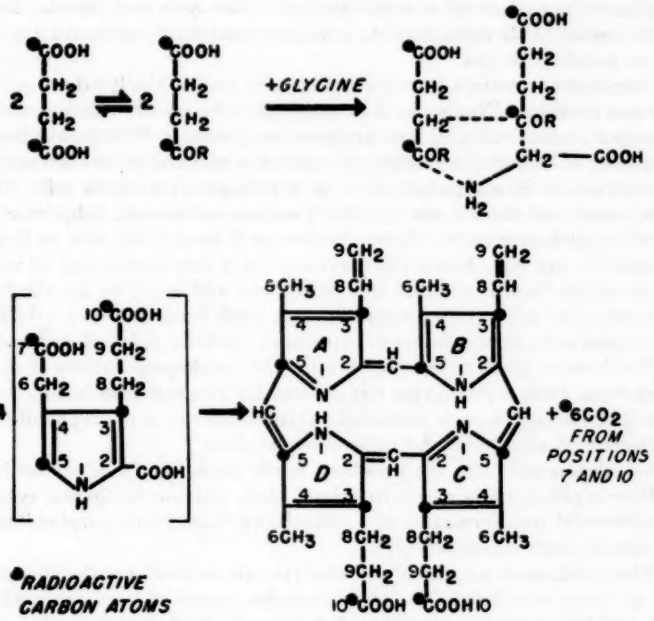
by Shuster (119a), one of which is similar to that of Granick & Vanden Schrieck. Both are sensitive to 0.05 μM. Detection in bacterial cultures has already been mentioned (111).

Biosynthesis

Two factual summaries (21, 24) record the elucidation of the biosynthesis of porphyrins and haem up to the beginning of 1951 and 1955 respectively. Reviews by Lemberg (13) on "Porphyrins in nature," Granick (20) on "Metabolism of haem and chlorophyll" and Rimington (18) on "Porphyrins" cover a wider field. The Ciba Foundation Symposium volume (4) contains papers by Shemin and others, with discussions, and the most recent review on biosynthesis comes from Rimington (25).

It is well known that with the identification of glycine as supplying the N atoms of protoporphyrin (1) and the discovery that acetate also contributed carbon atoms, Wittenberg & Shemin (120) devised a step-wise degradation procedure by which the activity of the individual carbon atoms of protoporphyrin could be determined. It had already been shown by Muir & Neuberger (121) and Wittenberg & Shemin (122) that the methine bridge carbon atoms are derived from the methylene carbons of glycine molecules. Degradation of haem synthesized in the presence of carboxyl-labelled and methyl-labelled acetate respectively (123) demonstrated that all remaining twenty-six carbon atoms of the porphyrin ring stemmed from acetate, almost certainly by way of an unsymmetrical four-carbon compound, possibly a derivative of succinic acid. The biosynthesis of protoporphyrin was thus brought into relation with the tricarboxylic acid cycle and confirmation was obtained by using as substrates ^{14}C-labelled succinate [Shemin & Kumin (124)], α-ketoglutarate and citrate [Wriston, Lack & Shemin (125)]; in each case the theoretically predicted distribution of labelling was observed. The stimulating action of crude co-enzyme A upon the synthesis from succinate suggested that succinyl-co-enzyme A might be the unsymmetrical derivative mentioned earlier.

The equivalence of labelling in corresponding positions of the four pyrrole rings of the porphyrin (123) and the incorporation of the intact succinate moiety suggested to Shemin & Kumin (124) that condensation of two molecules of succinate with one of glycine might give a common pyrrolic precursor which they formulated as is shown in Figure 2 on page 569. The postulated precursor would have had to lose the α carboxyl group since the carboxyl group of glycine is not incorporated into the porphyrin ring. Moreover, glycine methylene-carbon atoms would have, in some way, to provide the methine carbon atoms linking the pyrrole units into one macroring. About this time, Westall (69) isolated porphobilinogen from the urine of a patient with acute porphyria. Cookson & Rimington (70) showed that it was a monopyrrole with acetic acid and propionic acid side chains in the β positions and an aminomethyl group attached to one α position (Fig. 1). The structure is closely similar to that postulated by Shemin & Kumin (124)



The distribution pattern of C^{14} in protoporphyrin synthesized from C^{14}-carboxyl-labeled succinate.

FIG. 2. Hypothetical pyrrole precursor of rings A, B, C and D. [Shemin & Kumin (124)]

for their "common pyrrolic precursor." Falk, Dresel & Rimington (126) demonstrated that porphobilinogen added to chick red-cell haemolysates was utilized as a substrate for the production of uroporphyrin, coproporphyrin and protoporphyrin while almost simultaneously Bogorad & Granick (127) demonstrated porphyrin production from porphobilinogen by frozen and thawed *Chlorella* cells and cell extracts. Soon afterwards, Schwartz (128) demonstrated its conversion to copro- and protoporphyrins by homogenates of rat liver. Later researches by Dresel & Falk (129), Granick (130) and others (4) have amply confirmed porphobilinogen as a normal intermediate in haem synthesis and as the common pyrrolic precursor from which all four pyrroles of the porphyrin ring are elaborated. Almost simultaneously with the recognition of porphobilinogen as a porphyrin precursor (126), Shemin & Russell (131) showed conclusively by ^{15}N and ^{14}C labelling experiments that δ-aminolaevulic acid incubated with duck blood was incorporated into the haem formed; they indicated the manner in which two molecules of δ-aminolaevulic acid might condense to form porphobilinogen and also how the acid might participate in a succinate-glycine cycle. α-Amino-β-keto-

adipic acid is postulated as a participant in this cycle and Shemin, Russell & Abramsky (132) state that its intraperitoneal injection in the rat gives rise to porphobilinogen.

Porphyrin formation from δ-aminolaevulic acid in the lysed red cell system was confirmed [Neuberger & Scott (133)] and its conversion into porphobilinogen demonstrated in such preparations [Dresel & Falk (134).] Shemin, Abramsky & Russell (135) achieved conversion of δ-amino laevulic acid into protoporphyrin in a supernatant from centrifuged lysed duck cells. Thereafter, studies of the enzyme system "δ-aminolaevulic acid dehydrase" followed in quick succession. Gibson, Neuberger & Scott (136) used ox liver for its isolation but have found the enzyme to be widely distributed in nature. In the active form it contains free SH groups and is highly specific for its substrate. Granick's (130) preparation was made from chicken erythrocytes but similar activity was found in extracts of *Chlorella*, spinach, and a strain of *Tetrahymena geleii*. Schmid & Shemin (137) made preparations of the enzyme from duck erythrocytes but showed its presence also in human red cells. The conversion of two molecules of substrate to one of porphobilinogen was unequivocally proven by radioactive labelling.

δ-Aminolaevulic acid has thus been firmly established as a normal intermediate in porphyrin synthesis in animal, plant and microbiological systems. The chemical transformation of δ-aminolaevulic acid into porphobilinogen has already been mentioned (77).

The mechanism by which uroporphyrin arises from porphobilinogen is not yet fully elucidated. The transformation occurs not only enzymically (92), yielding uroporphyrin III and pseudouroporphyrin (also a III series porphyrin) but also spontaneously in acid or alkaline solutions. In an acid medium the product is predominantly uroporphyrin III but other isomers are also present; in alkali, on the contrary, a considerable proportion of uroporphyrin I is formed. Cookson & Rimington (70) who made these observations propose a reaction mechanism, based on electrochemical considerations to explain the production of the unsymmetrical III series iosmer from four porphobilinogen molecules. Others have postulated formation of an intermediate tripyrrylmethane [Shemin, Russell & Abramsky (132)] which by fission yields two different dipyrrylmethanes; porphyrin III formation is then explained by combination of a pair of these molecules. There is no obvious reason, however, why other pairs should not, at the same time, combine to give isomers I, II and IV. These authors state that formaldehyde is formed during the cyclization [confirmed also by Falk (4)] and this would appear to lend support to the scheme of Cookson & Rimington [(70) see also discussion in (4)]. Bogorad & Granick (127) propose a more elaborate mechanism (Fig. 3) involving formation at one stage of a branched tetrapyrrylmethane "T." While this has attractive features, it is as yet unsupported by evidence that such intermediates exist. Important work upon the enzymes concerned in porphyrin formation from porphobilinogen is reported by Bogorad & Granick (138) and Bogorad (139). A porphobilinogen "de-

aminase" may initiate the transformation. Then a substance is formed having high absorption at 500 mμ which may or may not be an intermediate on the direct line.

The effect of heat treatment upon *Chlorella* extracts leading to formation of uroporphyrin I in place of III (127) and a similar response by red-cell haemolysates [Booij & Rimington (140)] should help in the clarification of this problem. Stich & Eisgruber (141) suggest, from work on "yeast porphyria" that riboflavin is connected with the mechanism by which the synthesis is normally steered to produce principally the III series isomers. Fallot

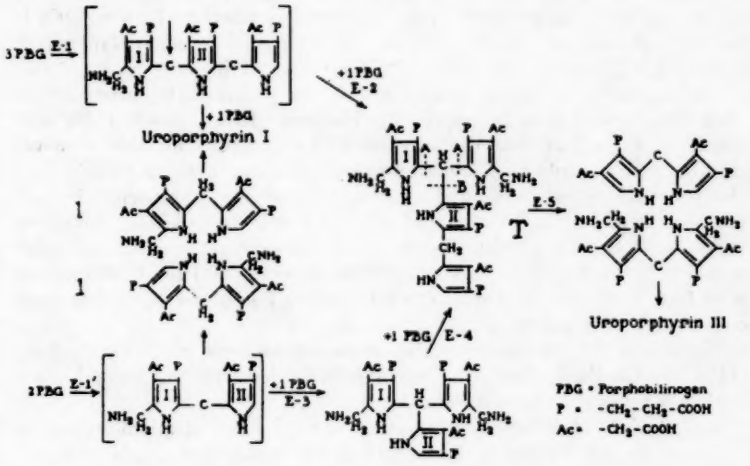


FIG. 3. Hypothetical scheme of porphyrin formation from porphobilinogen. Bogorad & Granick (127).

et al. (142) record that human blood incubated anaerobically with δ-aminolaevulic acid and glutathione produces only uroporphyrin I.

Whether or not the porphyrins lie on the direct biosynthetic pathway from porphobilinogen to haem is still debated. Dresel & Falk (4, 143) consider it to be more likely that some closely related derivatives are the true intermediates. The evidence concerning the utilization of uroporphyrin, coproporphyrin and protoporphyrin by *in vitro* systems has been conflicting [Dresel & Falk (143); Schwartz (128); Eriksen (4); also discussion after Dresel's paper in (4)] and results may depend upon the extent to which particles in the different systems have been disintegrated. Eriksen's experiments upon the formation, under anaerobic conditions, of radioactive porphyrins in erythrocytes from phenylhydrazine-treated rabbits and the progressive disappearance of these porphyrins on further incubation in air

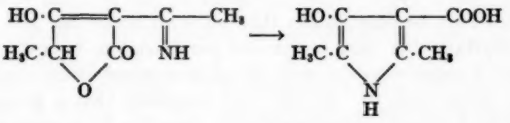
show that, in this system, porphyrins can be utilized. In 1952, Salomon, Richmond & Altman (144) showed from radioactive labelling experiments that uroporphyrin III occurs in rabbit bone marrow and is a precursor of protoporphyrin in that tissue. Possibly the conditions which these workers studied in the intact cells are such that porphyrins may be rapidly reconverted to the corresponding true intermediates while the reconversion is feeble or nonexistent in other more disintegrated systems. Eriksen (4) found both free and bound forms of protoporphyrin. The latter represented a later stage in utilization and deserves closer study; it may be of much significance in the process of iron-incorporation and the regulation of equivalence of globin and haem supply in the cell. One wonders whether the porphyrin is bound to globin, some precursor of globin [compare Schweiger, Rapoport & Schölzel (145)] or to the protein of the iron-incorporating enzyme.

Taking into account the finding of colorless intermediates between porphobilinogen and protoporphyrin by Bogorad (139) Granick (130) and others, and the fact that the schemes so far proposed for this chemical transformation would lead first to uroporphyrinogen, it seemed likely that the porphyrinogens corresponding to uroporphyrin, coproporphyrin and protoporphyrin might be the true biosynthetic intermediates [Rimington (18)]. As regards the first two evidence lending some support has been presented by Neve, Labbe & Aldrich (146) and Bogorad (139) and their findings have been confirmed in the writer's laboratory. The degree of utilization is not very great however.

Studies of the iron-incorporating system have been made by Granick (147) and Goldberg (148). The mechanism is definitely enzymic; further work will be awaited with interest.

Porphyrin and chlorophyll biosynthesis in plants and micro-organisms has been actively studied with the general conclusion that chlorophyll biosynthesis follows the same pathway up to protoporphyrin as does haem in animal tissues [Granick (4, 20, 149); Bogorad & Granick (150); Granick, Bogorad & Jaffe (151)]. A series of most interesting studies by Granick and his colleagues of *Chlorella* mutants [Granick (152)] reveal the further stages in chlorophyll biosynthesis to proceed in the following sequence: protoporphyrin→Mg protoporphyrin→Mg vinylphaeoporphyrin a_5→protochlorophyll *a*→chlorophyll *a*. Lascelles (4, 153) has shown that the same early pathway is followed up to protoporphyrin in the synthesis of bacteriochlorophyll by *Rhodopseudomonas spheroides*. Biosynthesis of porphyrins from δ-aminolaevulic acid by the Harderian glands of mice has been demonstrated *in vitro* by Davidheiser & Figge (154). Observations upon the effects of various metabolic inhibitors upon haem synthesis are scattered and do not yet allow of precise interpretation; they are nevertheless important. Abbott & Dodson (155) found that 2,5-dimethylbenzimidazole and also the 2,6 isomer inhibit incorporation of ^{15}N-glycine into haem by erythrocytes incubated *in vitro* for 24 hours. Abbott, Dodson & Auvil (156) later found that

porphyrin production from δ-aminolaevulic acid was not inhibited. The reviewer feels that this is probably to be interpreted as action by the inhibitor upon protein (probably globin) synthesis in the whole cell in which haem and globin production do seem to be linked in some way. An association of Vitamin B_6 with haemoglobin production has long been suspected. Schulman & Richert (157) find that haem synthesis *in vitro* from glycine and succinate by haemolysates prepared from blood of pyridoxine-deficient ducklings proceeds at less than half the normal rate. It is accelerated by addition of pyridoxal-5-phosphate but not of pyridoxine, pyridoxamine or pyridoxal. With δ-aminolaevulic acid as substrate, normal rates of synthesis and no acceleration was observed, indicating that the essential factor is concerned with steps prior to formation of porphobilinogen. Similarly, pantothenate was shown to be concerned also with these early stages. Confirmation of the essential nature of pyridoxine for erythropoiesis in the rat comes from Dinning & Day (158) while a first case of pyridoxine-deficiency anaemia in man has also been reported (159). Using avian red cells incubated with adenosine, glycine and acetate, Larsen & Orten (160) find that sedormid decreased production of all porphyrins except uroporphyrin while all porphyrins are decreased in the presence of isoniazide. 3-Acetimido-5-methyltetronic acid was found to increase porphyrin production. This is known to inhibit chlorophyll production in plants [Hamner & Tukey (161)] but to stimulate haem synthesis (4). As suggested by Rimington (4) it might rearrange into a substituted pyrrole, thus:



The effect of a number of substances including pterines, vitamin B_{12}, NaF, ATP, AMP, DPN, DNP, etc. on haem synthesis by lysed chick cells has been studied by Dresel & Falk (162). They have also studied the action of lead (129). Studies on NaF are reported by Bénard, Gajdos & Gajdos-Török (163), showing a stimulation of porphyrin production from glycine by phenylhydrazine-poisoned rabbit's blood and an inhibition in the case of normal chicken cells. Richmond, Altman & Salomon (164), working with rabbit bone marrow, considered the inhibition by malonate, α-ketoglutarate and ATP of haem synthesis from glycine to be due to interference with porphobilinogen formation. Gajdos & Gajdos-Török (4), however, find inhibition also at the porphobilinogen-porphyrin stage by these substances added to extracts of many different organs; AMP is stimulatory as are also phloridzoside and monoiodoacetate. Fluoride was often slightly stimulatory. KCN (0.005 M) and novarsenobenzole inhibit strongly.

Porphyria

Only those papers will be mentioned which contain matter of particular biochemical interest or which survey the subject from a particular point of view. Watson's school has contributed several reviews (8, 165 to 168). Markovitz (169) reviewed the literature up to the end of 1954. There were surveys by Rimington (9, 18) in 1952 and 1955, and by Schwartz (170) in 1953 on clinical aspects of porphyrin metabolism. Sunderman & Sunderman (171), in 1955, propose a modified classification in a well-documented review; Kark (172) in 1955 provides a very good clinically orientated review. There were summaries by Muir (173) in 1954, paying special attention to biochemical features; and by Gray (174) in 1956. The unusually high incidence of porphyria in South Africa is the subject of several papers by Barnes (175), Barnes & Marshall (176), Dean (177), Barnes (178) and Dean (179). The last paper reports a clinical genealogical study of 32 porphyria families in which 324 members had clinical manifestations. Detailed study of one of these families is recorded by Dean & Barnes (180). Waldenström (181) has collected data on all 321 known cases of acute porphyria in Sweden and is able to calculate a sex ratio of two males to three females and a frequency for the disease of 1.4 per 100,000 for the whole country but there is a much higher incidence in Lapland, where four large families reside. Mendelian dominant inheritance is confirmed.

A clear understanding of the porphyrias, from the chemical pathological point of view, is handicapped by the confusion surrounding the classification of the various clinical types. While discussion of this problem would be out of place here, it may be pointed out that some authors (166, 169, 179, 180) consider porphyria (other than congenital porphyria) to be a single disease entity capable of manifestations with either abdominal or cutaneous symptoms or both. Rimington (9, 18, 182, 183) considers that a group may be distinguished having high faecal porphyrin excretion in remission, and cutaneous symptoms only. In severe relapse, however, these may be accompanied by abdominal or nervous symptoms and even the excretion of some porphobilinogen. Only precise knowledge of the biochemical defects involved will clarify the situation. That different types do exhibit different biochemical pictures is exemplified by the studies of Schmid, Schwartz & Watson (184), Bolgert, Canivet & Le Sourd (185), and Canivet, Fallot & Poidatz (186), and others.

Congenital porphyria has been reported in swine and cattle by Jörgensen & With (187) in Denmark. Schmid, Schwartz & Sundberg (188) describe an abnormality of the normoblasts seen in five human cases of the disease; their summary of the world literature revealed only 34 cases with an acceptable diagnosis. Berger & Goldberg (189) describe a family with hereditary coproporphyria. Studies of porphyrin metabolism in cases of congenital porphyria are reviewed by Gray (190), those in a case of "mixed" type by Lowry, Hawkinson & Watson (191). Granick & Vanden Schrieck (119) showed that δ-aminolaevulic acid accompanied porphobilinogen in the urine of a case of

acute porphyria while Mauzerall & Granick (116) detected traces of both these substances in normal urine. The metabolism and excretion of δ-aminolaevulic acid has been studied by Berlin, Neuberger & Scott (192). Its ingestion by man is followed by transitory photosensitivity [Berlin *et al.* (193)] and Jarrett, Rimington & Willoughby (194) demonstrated porphyrin in the epidermis and formation of sub-epidermal blisters in rats which had been irradiated after its administration. α-Amino-β-ketoadipic acid, postulated by Shemin as a precursor of δ-aminolaevulic acid, gives rise to both porphobilinogen and porphyrins in biological systems [(132) Neuberger, Scott & Shuster (195)]. Enzymes which transform porphobilinogen into porphyrins are present in many tissues [Gajdos & Gajdos-Török (4)] and its metabolism and excretion have been studied by Goldberg & Rimington (196), Goldberg (197), Formijne & Poulie (198), Schwartz (199) and others. Watson (114) has reported porphobilinogen excretion in several conditions other than porphyria. Since it is a normal urinary constituent (116) one may have to recognize symptomatic porphobilinogenuria similar to the symptomatic uroporphyrinuria described by With & Petersen (200). Nevertheless, chromatographic confirmation of identity would seem desirable in view of the many Ehrlich-reacting substances which can be present in urines (117).

Examination of carefully purified porphyrins and porphobilinogen by Goldberg, Paton & Thompson (201) reveals no activity which could account for the symptoms of acute porphyria; similarly δ-aminolaevulic acid appears to be pharmacologically inert except for the photosensitizing effect, noted already (193, 194) of secondarily produced porphyrins. Gibson & Goldberg (202) have given a detailed description of the neuropathological changes seen in acute porphyria.

Experimental porphyria.—A finding of great importance is the discovery by Schmid & Schwartz (203) that allylisopropylacetylcarbamide ("sedormid"; APAC) administered to rabbits causes a disturbance of porphyrin metabolism chemically similar to human acute porphyria, with excretion of porphobilinogen and large quantities of porphyrins. The muscular weakness and dilatation of the stomach noted are not necessary consequences of the upset in pyrrole pigment metabolism since allylisopropylacetamide, which is devoid of hypnotic properties, was found by Goldberg (204) and Goldberg & Rimington (113) to produce an identical chemical picture but no paresis of the muscles. While the exact mode of action of these drugs is not yet clear, there can be little doubt that they will help in an understanding of human acute porphyria. In the latter, the liver is the seat of disturbance, so that the urinary porphyrins are mainly type III isomers (113, 203) and the porphobilinogen is identical with that found in the human disease [Brockman & Gray (205)]. Sedormid causes a rapid fall in liver catalase interpreted as a block to its formation [Schmid *et al.* (206); Schmid, Figen & Schwartz (207); Schmid & Schwartz, (4)]; other haem enzymes of the liver were affected only insignificantly. Perhaps related to the decrease in catalase is the accumulation of "green porphyrins" in the livers of experimental

animals [Schwartz (199); Schwartz & Ikeda (4)]. It is worth noting that no lowering of liver catalase was found by Gray (208) in a patient who died from acute porphyria. It would be interesting to test for porphyrinuric action other drugs depressing liver catalase, such as 3-amino-1,2,4-triazole [Heim, Appelman & Pyfrom (209)].

Pharmacological studies with drugs related to APAC have been carried out chiefly by Goldberg & Rimington (113), Stich (210), Stich & Decker (4, 211) and Talman, Labbe & Aldrich (212). Goldberg (213) also investigated a series of barbiturates and found that those bearing allyl groups such as dial and sodium alurate caused excretion in rabbits of both porphobilinogen and porphyrins; others caused porphyrinuria only or had little effect. From these experiments Goldberg & Rimington (4, 113, 204) defined the chemical structure necessary to cause experimental porphyria as follows:

$$CH_2{=}CH{-}CH_2{-}\underset{\displaystyle R}{CH}\cdot CO\cdot NHR'$$

Stich & Decker (4, 211) came to essentially similar conclusions. Gajdos & Gajdos-Török (4) find the action of these drugs to be very rapid and believe porphobilinogen production takes place in other organs as well as the liver. Formijne & Poulie (214) believe that a precursor of porphobilinogen may also be excreted in the urine of intoxicated animals.

The chick embryo responds to APAC as do rabbits [Talman *et al.* (215)]. This group has shown a depression in purine synthesis in APAC-treated eggs coincident with the rise in porphyrins and also that livers of rabbits with experimental porphyria exhibit reduced utilization of 4-amino-5-imidazolecarboxamide (216). They suggest that the site of inhibition in the succinate-glycine cycle may be the oxidative deamination of δ-aminolaevulic acid.

Urinary Porphyrin Excretion

The presence of uroporphyrin in normal urine, demonstrated chromatographically by Nicholas & Rimington (90), has been confirmed by Lockwood (217), who gives a range of 15–30 μg. per day, and Schwartz (170). It is the I series isomer together with a trace of uroporphyrin III [Lockwood & Bloomfield (218)]. That the quantity may increase in several diseases other than porphyria is reported by With & Petersen (200). Rimington & Sveinsson (81) proposed a spectrophotometric correction formula for use with the method of Sveinsson, Rimington & Barnes (88) but Dresel, Rimington & Tooth (85) have now described a direct extraction method for determining urinary uroporphyrin, based on the cyclohexanone solubility observed by Kennedy (219). An improved method for urinary coproporphyrin determination is described by Schwartz, Zieve & Watson (79) and normal values for men and women are given as 166 ± 45 μg, and 134 ± 42 μg per day respectively (80). Normal urinary and faecal coproporphyrin values for rats are given by Hoffbauer, Watson & Schwartz (220). They (221) find injected

coproporphyrin III to be excreted via the bile with none in the urine unless the bile duct is occluded. Animals poisoned with CCl_4 excreted about equal amounts in faeces and urine. Comfort & Weatherall (222) find lead-poisoned rabbits excrete coproporphyrin III in the urine but largely as a precursor with only a trace, if any, of the I isomer. Intravenous injection into either normal or lead-treated rabbits resulted in excretion of 35 to 75 per cent of the dose in the bile and none in the urine. It is suggested that the metabolic disorder produced by lead leads to overproduction or under-utilization of a precursor of coproporphyrin III which is excreted entirely as such in the urine and thereafter suffers gradual conversion into porphyrin, [Weatherall (223)]. Kench, Lane & Varley (224), however, find about 30 per cent of the coproporphyrin in human cases of lead poisoning to be the I series isomer.

That coproporphyrin was excreted in urine largely as a precursor, converted to porphyrin by ultraviolet irradiation or more efficiently by oxidation with dilute iodine, was established by Watson *et al.* (225). The chromogen was extracted both by ether and by ethyl acetate although the somewhat higher total porphyrin values obtained using ethyl acetate are considered by Holeček (226) to be due to better solubility of the chromogen in this solvent. Isomer analyses on porphyrin excreted as chromogen have revealed the same ratio in any given sample of urine as that found in the preformed coproporphyrin. Only small proportions of chromogen are demonstrable in faeces. Shiels and his co-workers (227) again show that urinary coproporphyrin is a reliable and sensitive test for intoxication in persons exposed to lead hazards. There is good correlation between the urinary porphyrin and blood lead levels. Coproporphyrinuria is constantly seen after myocardial infarction [Koskelo (228)].

In the normal subject, total urinary coproporphyrin excretion appears to be proportional to body weight [Strait *et al.* (229); Hsia & Page (230); Neve & Aldrich (231)]. In cutaneous porphyria, Schück & Berman (232) find a parallel between creatinine and porphyrin excretion in urine and conclude that porphyrin is eliminated as a glomerular filtrate thus permitting calculation of plasma porphyrin concentrations from urinalysis. Porphobilinogen is eliminated in a similar manner [Goldberg & Rimington (196)].

Porphyrins and Cancer

Much interest has developed concerning a possible association between malignancy and porphyrin content of tissues, the ability of neoplasms to concentrate injected porphyrin and also the manner in which haematoporphyrin sensitizes paramoecia to x-rays. Data bearing on these topics have been summarized by Schwartz, Absolon & Vermund (233). Bingold, Stich & Cramer (234) find that haemoprotein enzymes such as catalase and cytochrome *c* are much reduced in tumor tissue and that with few exceptions porphyrins are completely absent. From this they conclude that there is in malignant cells an inability to synthesize the tetrapyrrole system, a characteristic which may account for the lowered respiratory activity of tumors. Rasmussen-Taxdal, Ward & Figge (235) observed that intravenously in-

jected haematoporphyrin is rapidly taken up by tumor cells and is thus of help to the surgeon in localizing primary and secondary growths at operation. That such avidity for haematoporphyrin is common to all rapidly growing tissues, normal or neoplastic, has been demonstrated by Kennedy (236). Figge & Wichterman (237) also found the addition of 1/500,000 haematoporphyrin to suspensions of paramoecia renders them about twenty times more susceptible to the lethal effects of x-irradiation. Of interest in connection with the cancer problem is the note by Treibs (238) reporting synthesis of a di-iododeuteroporphyrin containing ^{131}I.

Cytochromes

Certain findings of outstanding interest may be noticed. In his Nobel Prize Lecture, Theorell (239) indicated the probable association between haem groups, cysteine, and histidine residues in the α-helix of cytochrome *c*. Morrison, Eastbrook & Stotz (240) find evidence for two different haems in this protein. Crystalline cytochrome *c* has been obtained by Bodo (241) from King penguin muscle and by Hagihara and colleagues (242) from yeast and from hearts of the ox and pig. The prosthetic group of cytochrome a_2 from *Aerobacter* and *Azotobacter* is considered by Barrett & Lemberg (243) to be chlorin-like with a lipide group but neither CO nor CHO function. Lemberg and colleagues (244) have carried further the purification of porphyrin *a* which is considered to be the prosthetic group of both cytochromes *a* and a_3 (cytochrome oxidase). Cryptoporphyrin *a* is probably an artifact. Difficulties in acceptance of the structure for porphyrin *a* proposed by Warburg & Gewitz (245) are advanced by Lemberg and his group. It is now generally accepted that a long alkyl side-chain explains the lipophilic character of porphyrin *a*, and that it is not a phytyl ester. A new soluble cytochrome is reported by Elsden, Kamen & Vernon (246).

Drabkin (247) gives extensive data on the quantitative distribution of haemoglobin, myoglobin and cytochrome *c* in tissues of different species and their relation to body weight. He has also investigated the biosynthesis of cytochrome *c* in normal and regenerating tissues and the effects of the endocrine system on the rate of biosynthesis (4, 248, 249). Vannotti (4) reported experiments in the same field.

Finally the reviewer would mention the isolation by Appleby & Morton (250) of the lactic dehydrogenase, cytochrome b_2, in crystalline form and its recognition as a haemo-flavoprotein. This is a most interesting development in our knowledge of cellular catalysts. The implications of the discovery and the possibility of other enzymes occurring in which two prosthetic groups are bound to the same protein carrier are discussed in an article by Morton (251). The haemo-flavo-protein nature of cytochrome b_2 has been confirmed by Boeri *et al.* (252). There is one residue each of haem and flavin per molecule. A less well-defined enzyme preparation, the DPN-oxidase from ox heart, was reported by Mackler *et al.* (253) to contain approximately 1 flavin and 5 haem residues, 10 non-haemin atoms of iron and 1 of copper. One of the

haems resembled protohaem, the other was a "green haemin" with absorption at 607 mμ. The molecule also contained much lipide.

Miscellaneous

The occurrence of a porphyrin, probably uroporphyrin, is reported for the first time in a planarian, *Dugesia dorotocephala*, by Krugelis-Macrae (254); these animals are light-sensitive. A strain of *Tetrahymena geleii* develops much protoporphyrin at the end of its rapid growth phase on solid media in darkness [Rudzinska & Granick (255)]. In an examination of some British marine invertebrates Kennedy & Vevers (256) report the presence of coproporphyrin III in the annelids *Nereis diversicolor*, *Chaetopterus variopedatus*, and *Myxicola infundibulum*, of uroporphyrin I in two molluscs, *Aplysia punctata* and *Duvaucelia plebeia*, and of protoporphyrin in the integument of the echinoderms *Astropecten irregularis*, *Luidia ciliaris* and *Asterias rubens*. In the first two, chlorocruoroporphyrin was also present. Bacteriochlorophyll *a*, the pigment of purple bacteria, is described by Seybold & Hirsch (257). Crystallization of both chlorophylls *a* and *b* and of bacteriochlorophyll is described by Jacobs, Vatter & Holt (258).

From Kosaki and his collaborators (259) have come a series of papers dealing with porphyrins in different tissues, their recognition by fluorescence and the factors influencing the natural porphyrin content or the amount taken up from applied protoporphyrin solutions. Normal and pathological tissues are also compared in these respects. Certain azaporphine derivatives (phthalocyanins) have been used as histological stains for nervous tissue by Klüver & Barrera (260). Hartree (261) has compiled methods for qualitative and quantiative analysis of materials for cytochromes, peroxidase, catalase and root-nodule haemoglobin. The porphyrin derived from lactoperoxidase probably contains an acetyl side-chain [Morell (262)].

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CLINICAL APPLICATIONS OF BIOCHEMISTRY[1,2]

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This review deals with the chemical alterations observed in disease, especially those relating to chemical mechanisms. The discussion of methods has been omitted except in a few special instances, since the development and application of analytical methods is reviewed biannually in *Analytical Chemistry* (1, 2). Progress in the chemical study of disease has been so extensive that it has not been possible to include all of the important developments because of limited space.

Disturbances of Adrenal Function

Aldosteronism.—Conn (3) reviewed the occurrence of this syndrome which in its primary form may be due to adenoma or hyperplasia (4, 5) of the adrenal cortex, and may be congenital (6). Hypersecretion of aldosterone occurs also in a secondary form when diets low in sodium and rich in potassium are fed (7), and in the nephrotic syndrome (8), congestive heart failure 7, 9, 10), malignant hypertension (11), cirrhosis of the liver (7, 12, 13, 14), epidemic hepatitis (13), pregnancy (7, 15, 16), and after surgical operations (17). The increased excretion in severe liver disease may be the result of impaired inactivation of the liver [Chart *et al.* (19)]. High aldosterone excretion was found in a patient with previously unexplained edema of 20 years duration (18).

Some patients with adrenocortical tumors may show many of the characteristic symptoms of aldosteronism, the excessive losses of K and Mg in urine and feces, low serum K, and alkalemia, and yet not excrete increased amounts of aldosterone in their urine [Chalmers *et al.* (20)]. On the other hand, as Tait (21) pointed out, much larger amounts of aldosterone often are found in the urine in cirrhosis, etc., than in patients with primary aldosteronism. To be sure, some uncertainty exists concerning the adequacy of present methods for aldosterone assay. Venning & Dyrenfurth (15) increased the yield of aldosterone by using acid hydrolysis following hydrolysis with beta glucuronidase. However, the pregnancy urines they studied were especially rich in conjugated aldosterone. Venning *et al.* (23) found, by means of bio-

[1] The literature reviewed includes that of November, 1956.

[2] The following abbreviations are used in this chapter: 5-HIAA for 5-hydroxyindoleacetic acid; HLD for hepatolenticular degeneration (Wilson's Disease); SGOT for serum glutamic oxalacetic transaminase; and SGPT for serum glutamic pyruvic transaminase.

[3] The author is indebted to the University College of the West Indies, Jamaica, British West Indies, for making available library and other facilities that aided the preparation of this review.

assay, 3.2 and 3.8 μg. per 24 hr. in men and women. Neher & Wettstein (26, 27) found by a chemical method up to 10 μg. per 24 hr. in adults. Ayres, Simpson, & Tait (24) reported a mean of 10.5 μg. per 24 hr., using a physicochemical method. Moolenaar's (25) method gave similar results.

It appears unlikely that analytical factors could completely account for the discrepancies, and it is possible that a new urinary steroid related to mineral metabolism, "wildegosterone," isolated by Brooks, Mills, & Prunty (22), may provide an explanation. This steroid was found in the urine of a patient with the syndrome of aldosteronism but without elevated aldosterone excretion. A second patient excreted increased amounts of both steroids. A sharp peak at 238 mμ serves for the recognition of wildegosterone which is thought to have a Δ4-3 ketone configuration. Increased amounts were found in urine in pregnancy.

The excretion of aldosterone is closely correlated with intake and excretion of sodium (28, 29). A relatively small increase in body sodium greatly decreased aldosterone excretion. Duncan *et al.* (29) proposed that the decrease in urinary aldosterone reflected a similar change in circulating aldosterone. Change in the latter then leads to the increased excretion of sodium. Although the relationships between sodium and aldosterone excretion were qualitatively similar in heart failure and hepatic cirrhosis, the aldosterone excretion did not fall as low and the sodium excretion did not rise as high as in normal subjects. Such an attenuation of response might account for the formation of edema.

Muller *et al.* (30) and Bartter *et al.* (31) found that the homeostatic mechanism involved changes in volume of body fluid rather than of Na^+ or K^+. The latter (31) concluded that increased intracellular fluid volume, regardless of change in extracellular water, was the physiological change immediately related to decreased aldosterone excretion. Conversely, when extracellular fluid volume was decreased, an increased output of aldosterone resulted. Changes in circulating or total K were excluded as mediators of the volume control of aldosterone secretion. However, Mulrow *et al.* (28) concluded that the urinary K/Na ratio was the parameter directly correlated with aldosterone excretion. The correlation was so close that the K/Na ratio could be used for estimating aldosterone excretion.

Although Muller *et al.* (32) showed that corticotrophin caused some rise in aldosterone secretion, this was far less than that of hydrocortisone. It seems clear that the two are primarily regulated by different mechanisms (33).

Adrenocortical function.—Improved procedures for the diagnosis of altered states of adrenocortical function were described by Laidlaw *et al.* (34). They depend upon measurement of 17 keto- and hydroxy-steroid excretion in urine and of circulating eosinophiles in the blood following intravenous administration of corticotrophin. The absence of any response was characteristic of Addison's disease. Patients with hypopituitarism or iatrogenic adrenocortical insufficiency responded with increased urinary steroid excretion. A hyperactive response occurred in the adrenogenital syndrome, in

contrast to a normal one in Cushing's syndrome. The closely allied problem of the study of adenohypophyseal function has been discussed by Crooke (35).

Pheochromocytoma.—Large amounts of 3-hydroxytyramine and 3,4 dihydroxyphenylalanine, in addition to epinephrine and nor-epinephrine, were found by Weil-Malherbe (36) in a pheochromocytoma. The urinary excretion also was high and fell after removal of the tumor. The excretion of 3-hydroxytyramine at times exceeded that of nor-epinephrine. McMillen (37) also found a substantial content of 3-hydroxytyramine in a paraganglionic tumor.

Aminoacidurias

Hartnup or "H" syndrome.—A detailed report of this interesting aminoaciduria, familial in occurrence, is given by Baron *et al.* (38). Formerly known as Hart's syndrome, it is characterized by pellagra-like skin rash and cerebellar ataxia. It has been shown that some of the pellagrins discovered within recent years in the United Kingdom were actually suffering with the Hartnup syndrome (38). Up to 1100 mg. of amino acid were excreted daily, the normal excretion in controls being 40 mg. A specific pattern of amino acid excretion was defined. Indole compounds also were found in the urine in abundance, including indican, indolylacetic acid (50 to 200 mg. daily), an unidentified indolic substance, and tryptophan. The feces contained large amounts of protoporphyrin. The underlying cause may be an interruption in the sequence of reactions between tryptophan and nicotinamide. A heavy loss of tryptophan and its metabolites thus may contribute to the symptomatology, as may a lack of nicotinamide. A second child in the same family developed abnormalities while the first was being studied. Two other siblings had marked aminoaciduria and two were apparently normal.

This and other aminoacidurias were studied by Evered (39) with the aid of the chromatographic technique of Moore & Stein (40). Concentrations of several amino acids were measured simultaneously in blood and urine, and clearances calculated from these data indicated that no tubular reabsorption of amino acid occurred in some instances. Evered also studied the osteomalacia associated with aminoaciduria, phosphaturia, low renal threshold for phosphate, and low plasma phosphate, a syndrome discovered by Astrup & Dent (41). Enormously increased excretions of glycine, proline, and methyl histidine were found. The high concentrations in the renal tubular contents are believed to depress phosphate reabsorption and so contribute to the osteomalacia. Aminoaciduria was found in pernicious anemia (42) but not in other types of megaloblastic anemia. Adequate treatment with vitamin B_{12} caused it to disappear (43).

Serum Bilirubin and Hemolytic Disease of Newborn

Bilirubin and conjugated bilirubin.—Overbeek *et al.* (44) found that bilirubin in bile, when treated with diazotized sulfanilic acid, formed a different

and more polar dipyrryl azo pigment than did bilirubin. Billing & Lathe (45) and Schmid (46) found that the biliary bilirubin which reacted promptly in aqueous solution with the diazo reagent yielded on hydrolysis sufficient glucuronic acid to prove that most of the bilirubin was present as the diglucuronide. On the other hand, it has been known that bilirubin reacts readily with the diazo reagent only in the presence of ethanol or other accelerant. Thus, a satisfactory explanation for the observations made by van den Bergh & Müller (47) 40 years ago is finally available. Lathe (48) proposed that the old terminology of "direct" and "indirect-reacting" bilirubin be replaced by the more descriptive "conjugated bilirubin" and "bilirubin." Talafant (50) also identified direct bilirubin as a conjugate of glucuronic acid. A monoglucuronide of bilirubin also is present in bile and serum (45). Klatskin & Bungards (49) effectively demolished the hypothesis that the different diazo reactions of bilirubin depended upon combinations with globin or α_1-globulin. All, or very nearly all, of each form of bilirubin in serum is combined with albumin.

The transformation of bilirubin into the glucuronide appears to be an essential step in its secretion into bile, since only a small amount of bilirubin exists in bile. This could be formed by spontaneous hydrolysis of the glucuronide (45). The glucuronide is virtually absent from serum of the newborn possibly because the conjugating mechanism is not yet functioning in the immature liver. Higher bilirubin concentrations exist in the serum of the premature infant than in those born at full term (50, 51). One of the contributing causes was the use of unnecessarily large dosages of vitamin-K analogue (52, 53). Whether bilirubin is responsible for the brain injury often associated with hemolytic disease of the newborn remains uncertain, although circumstantial evidence incriminating it is impressive. Yet, no predictable correlation was found between the bilirubin content of plasma and cerebrospinal fluid in hemolytic disease of the newborn (54, 55).

Additional studies have confirmed the presence of heme pigments in the sera of some newborn (255). Hemoglobin and methemalbumin have been identified (56, 57, 58). Methemoglobin probably is present also. These pigments not only complicate the accurate measurement of bilirubin in such sera, but, in addition, undoubtedly play a part in the development of the kernicterus syndrome. Penetration of bilirubin into the brain is not solely dependent upon its concentration in plasma. Tuttle (56) suggested that heme may compete with bilirubin for the binding sites of albumin. In the presence of much heme, fewer sites would be available to bilirubin so that more of the latter might penetrate into the tissues.

Argentaffin Carcinomas (Malignant Carcinoid)

Lembeck (59) reported that malignant carcinoid tissue, consisting of enterochromaffin cells, contained two to five mg. of 5-hydroxytryptamine (serotonin) per gm. This led Thorson *et al.* (60) to relate the secretion of this powerful vaso-active substance by these cells to the vasomotor disturbances,

intestinal hypermotility, bronchospasm, and valvular lesions of the heart that constitute the malignant carcinoid syndrome. Increased concentrations of serotonin were, in fact, demonstrated in urine and blood by Pernow & Waldenström (61) and Sjoerdsma *et al.* (62). However, a quantitatively more impressive abnormality found by Sjoerdsma *et al.* (63) and Page *et al.* (64) was the enormously increased excretion of 5-hydroxyindoleacetic acid (5-HIAA), an important metabolite of serotonin (80) in the urine.

Much of the tryptophan intake of patients with the malignant carcinoid syndrome is diverted to the production of serotonin (106). In such patients, as much as 60 per cent of ingested tryptophan appears in the urine as 5-HIAA, whereas in normal persons, only small amounts can be detected (62). Boyland (65) found 3 to 14 mg. of indoleacetic acid per day in the urine, in addition to serotonin and 5-HIAA. Successful removal of argentaffin tumor may cause the excretion of serotonin and its metabolites to return to normal (62, 65). However, metastatic activity is so prevalent that this response is not the usual one.

The typical syndrome develops only when there is a substantial bulk of tumor tissue, and large metastases to the liver are invariably present. Despite the large amounts of 5-HIAA excreted, relatively little serotonin is detected in peripheral blood. An explanation is offered by the experiments of Gable *et al.* (66) who found that two thirds of the free serotonin (not contained in the platelets) present in pulmonary artery blood was removed by passage through the lungs. These are known to be rich in the amine oxidase which is capable of converting serotonin into 5-HIAA [Blaschko & Philpot (67); Erspamer (68)]. Rapid destruction of serotonin was demonstrated also by Erspamer (69), who recovered only 27 to 46 per cent of serotonin injected into rats.

The lability of serotonin in the circulation, together with the ease with which it may be released into plasma from platelets, makes the usefulness of plasma serotonin assays uncertain for diagnosis (71). By contrast, 5-HIAA is present in much higher concentrations and several chromatographic methods have been proposed for its detection (65, 72 to 75) and measurement (72, 75). Heilmeyer *et al.* (70) used high voltage electrophoresis. Chromatography by a two-dimensional technique such as that of Jepson (74) enables the other indole substances often present in the urine to be detected. Udenfriend *et al.* (76) developed photometric and fluorometric methods. The excretion of 5-HIAA by healthy persons and by those with miscellaneous diseases was measured (62, 71, 77, 78). Although the diagnosis of malignant carcinoid was fairly obvious without chemical confirmation in nearly all patients so far described, the possibility that early stages of the illness might be detected and treated has encouraged the development of laboratory methods. Postoperative recurrences may be recognized by assays for 5-HIAA in urine. Alcohol (71) or fatty foods (79) precipitated the systemic disturbance and might be used in conjunction with chemical studies to provide a provocative test.

Chemotherapy with colloidal Au^{198} relieved some symptoms of malignant carcinoid (66), perhaps because it temporarily inactivated an inhibitor of the amine oxidase in the lungs. Chlorpromazine behaved as a serotonin antagonist [Benditt & Rowley (81)] and Cole & Bertins (82) reported that it ameliorated the diarrhea and certain other symptoms of a patient with the malignant carcinoid syndrome.

Excretion of serotonin, 5-HIAA, and indoleacetic acid by patients with carcinomas of the larynx and bronchus was significantly greater after two gm. of tryptophan by mouth than by patients with malignant neoplasms in other locations (65).

Disturbances of Copper Metabolism

Hepatolenticular degeneration (HLD; Wilson's Disease), according to Bush *et al.* (83), characteristically shows the following abnormalities of Cu metabolism: decrease in total plasma Cu concentration, decreased ceruloplasmin, increased direct-reacting Cu (a fraction probably bound to albumin), greatly increased urinary excretion of Cu, and increased tissue Cu. Recovery of radioactive Cu, given either by the oral or intravenous route, was much greater in urine of patients with HLD than in control subjects. Less of the Cu was recovered in ceruloplasmin, and the uptake by the liver was decreased. The excessive absorption of Cu from the gastrointestinal tract that caused Cu accumulation could be inhibited by use of K_2S. Uzman *et al.* (84) detected in the liver proteins in HLD, by electrophoretic studies, an extra component with a strong avidity for Cu. Excessive absorption of Cu from the gastrointestinal tract was attributed to this abnormality. A genetically determined defect in protein metabolism was believed to be the cause. A search for the mechanism responsible for the injury to the brain in HLD showed that Cu^{++} inhibited ATP dephosphorylation, and, temporarily, conduction in frog sciatic nerve [Hansl *et al.* (85)]. According to Ravin (86), the most consistently abnormal chemical finding in HLD was a lowered Cu oxidase activity of serum; he described a simplified method for its measurement.

Administration of penicillamine has been proposed by Walshe (87) for treatment of HLD. A large increase in Cu excretion, attributed to a chelating action, followed its use. Gros (88) found that injections of glutamic acid lowered serum Cu. Ceruloplasmin caused no striking change when tested as a therapeutic agent [Bickel *et al.*, (89)].

A Cu deficiency syndrome in infants characterized by low serum Cu, Fe deficiency anemia, and hypoproteinemia was described by Sturgeon & Brubaker (90). Butler & Newman (91) reported lower concentrations of Cu in serum and urine than those found previously by others. Rigorous precautions against contamination were held responsible.

Galactosemia

Impaired ability to metabolize galactose, a tendency to develop cataracts, and other serious disturbances of metabolism comprising the syndrome of

galactosemia were shown to be caused by the lack of a single enzyme, P galuridyl transferase [Kalckar, Anderson, & Isselbacher, (92)]. Schwarz *et al.* (93) and Komrower *et al.* (94) found that galactose-1-phosphate accumulated in the tissues in galactosemia and attributed this to lack of galactowaldenase. However, Isselbacher *et al.* (95) proved that the latter was present and active if sufficient diphosphopyridine nucleotide was added. They and Anderson (96) reported that blood of normal infants had a high P galuridyl transferase activity but that galactosemic infants had only 1/40 of the normal activity. A defect in the synthesis of the transferase was postulated.

Galactosemia is accompanied by an aminoaciduria typical with respect to kinds and amounts of the various amino acids excreted [Cusworth *et al.* (97)]; methionine was not found.

Glycogen Storage Disease

Generalized glycogen storage disease in which the glycogen has abnormally short outer chains was characterized by the absence of amylo-1,6-glucosidase (debranching enzyme) from skeletal and heart muscle [Illingworth *et al.*, (98)]. It is believed that this is a distinct type of glycogen disease. Yet another type, affecting the liver, with normal glycogen structure, and nearly normal glucose-6-phosphatase activity was described by Zetterström & Sörbo (99).

Hypoglycemias

A remarkable hypoglycemia, familial in occurrence, which was precipitated by the ingestion of protein or amino acids was described by Cochrane *et al.* (100). Leucine was particularly active in causing the blood sugar to fall. Low blood sugar concentrations were accompanied by greatly increased reduction due to substances other than sugars. Isovaleric acid, a product of leucine metabolism, was as active as leucine in causing symptoms. Both are thought to stimulate insulin secretion or to enhance its activity.

For more than 50 years, epidemics of "vomiting sickness" in Jamaica have had serious effects. In 1951 an outbreak affected 151 persons and caused 32 deaths in one locality (101). Stuart *et al.* (102) found it to be a manifestation of a severe epidemic hypoglycemia. Fatty changes in the liver with almost complete absence of glycogen were described [Patrick *et al.* (103); Hill *et al.* (104)]. A toxin from the fruit or seeds of the ackee tree (*Blighia sapida*) is held responsible and Hassall & Reyle (105) isolated two new polypeptides, hypoglycin A and B, from this source. Both produced fatal hypoglycemia in laboratory animals. Conn & Seltzer (108) have revised and enlarged the etiologic classification of the spontaneous hypoglycemias.

Insulin and Diabetes Mellitus

Plasma insulin activity, when measured by the rat diaphragm method of Vallance-Owen *et al.* (109), was much higher in obese diabetic patients than in normal persons (110). It increased in plasma, usually, after the adminis-

tration of glucose. The activity of insulin when added to normal plasma was undiminished; however, insulin added to the plasma of uncontrolled but nonketotic diabetics caused no rise in activity, apparently because of inhibition. It appears that many diabetics require insulin to overcome an inhibitor circulating in their plasma. This would explain why the requirement in diabetes mellitus often exceeds that following complete removal of the pancreas. Insulin antagonism in sera of patients with diabetic acidosis was detected also by Field & Stetten (111). The activity of the humoral antagonist was associated with alpha globulin and disappeared after treatment. Antagonist equivalent to 10,000 units of insulin was found in one patient's plasma. A study of labeled insulin by Welsh *et al.* (112) showed that it disappeared less rapidly from the plasma of insulin-treated diabetics or schizophrenics than from that of untreated controls. Entry of insulin into the tissues was presumably hindered by the presence of a binding agent which decreased its hypoglycemic action but also protected it against degradation. Zone electrophoresis of insulin labeled with I^{131}, using paper pretreated with insulin, located the "antibody" to insulin in the region of rapidly moving γ-globulin [Berson *et al.* (113)]. Thus it differs from that described by Field & Stetten. A preparation of γ-globulin from a patient with an extremely high insulin requirement and elevated γ-globulin concentration possessed marked anticonvulsive action when mixed with insulin. This property was lost after treatment of the patient with cortisone [Colwell & Weiger (114)].

Kelly (115) used plasma insulin assay to confirm a diagnosis of islet cell tumor of the pancreas. Although hypoglycemia was of minimal severity, plasma insulin activity was greatly increased.

Shreeve *et al.* (117) compared the oxidation of glucose in diabetes mellitus of varying severity with that of normal persons. The percentage of CO_2 derived from glucose decreased by nearly one half, whereas the glucose turnover doubled in the diabetics. Thus, decreased utilization and increased production of glucose were both demonstrated. Baumgarten (116) showed that pentoses, fructose, and unidentified sugars were present in urine, together with glucose, in increased amounts in diabetes mellitus. Lukens (118) reviewed the criteria for appraisal of the severity of diabetes.

Heart Disease

Measurement of the activity of serum glutamic-oxalacetic transaminase (SGOT) has become a standard procedure in the chemical laboratories of many hospitals within the past two years. Few patients with myocardial infarction fail to show a significant rise in SGOT within 24 hr., the incidence reported being 90 to 98 per cent, and its extent two to 20 times the normal level. Elevated values occurred as early as six hr., became maximal at 24 to 36 hr., and returned to normal three to five days after myocardial infarction [LaDue & Wroblewski and others (119 to 124)]. Little or no rise occurred in patients with pulmonary embolism and this may aid its differentiation from myocardial infarction (125); see also (124). Measurement of the activity of

SGOT may resolve ambiguities in electrocardiographic diagnosis of myocardial infarction (121, 123).

White (126) found serum lactic dehydrogenase to be more helpful than SGOT for confirming the presence of myocardial infarction because the elevated activity of the former persisted longer. Besides lactic dehydrogenase (127 to 131), increased activities of malic dehydrogenase (130, 131), aldolase (126, 130, 132), and hexose isomerase (126) have been demonstrated. Wacker *et al.* (131) found that the concentration of zinc in serum decreased following myocardial infarction, and Adelstein *et al.* (133) noted that serum Cu increased to maximal concentrations between five and 11 days after the infarction. The amount of Cu appearing in the circulation exceeded that expected if the injured myocardium was the sole source. Oxidase activity of serum, measured with the aid of benzidine and *p*-phenylenediamine substrates, also increased.

The failing heart uses its sources of energy less effectively than the normal heart despite slightly greater extraction of oxygen from the coronary flow [Blain *et al.* (134)]. Lactate extraction decreased only at higher arterial lactate concentrations. Consumption of glucose, pyruvate, fatty acids, ketone substances, and amino acids by the myocardium was unaltered in compensated heart disease or myocardial failure. Olson *et al.* (135, 136) found that myosin extracted from the heart in chronic congestive failure was more highly aggregated than that from the myocardium of normal dogs.

Liver Disease

Serum enzymes.—Greatly increased activities of SGOT occurred in viral hepatitis (137 to 140). High activities prevailed also in the presence of liver cell injury associated with invasion of the liver by cancer (142), and SGOT frequently gave the only indication of its presence. In cirrhosis, changes were less marked than in viral hepatitis, and this was true also of biliary obstruction (143). Bile was reported to contain large amounts (122). Enormously increased activities of SGOT followed intoxication with CCl_4 (141). SGOT offers promise as a tool for study of the epidemiology of viral hepatitis. A rise, occurring shortly after exposure to an infected person, persisted for weeks before the appearance of overt symptoms (139, 140).

Since elevated SGOT may occur in many diseases, serum glutamic-pyruvic transaminase (SGPT) was studied because of its predominance in liver [Wroblewski & LaDue (144)]. No rise in SGPT occurred in myocardial infarction. In liver disease the behavior of SGPT resembled that of SGOT (138, 144), although it was less responsive to some forms of liver injury and more affected by biliary obstruction (144). In hepatitis, activities of SGPT were higher than those of SGOT despite the lower concentration of the former in liver tissue (144). Thus the activity of transaminases in serum was not solely dependent upon release from necrotic liver cells. Other factors, for example gradients in functional activity within the liver lobule (146) and differences in rates of excretion, must be considered.

The use of acetylsalicylic acid for treatment of rheumatic fever was followed by a rise in the activities of both SGOT and SGPT after a delay of one to two weeks [Manso *et al.* (147)]. Wang & Appelhanz (148) found SGOT to be elevated after a meal.

Serum lactic dehydrogenase (128, 149) and aldolase (150, 151) also increase in liver disease, probably due to the same factors that effect the increase in the transaminases. Release of stores of Fe and ferritin from damaged hepatic cells would explain the increase in serum iron (152, 153), ferritin (154), and vitamin B_{12} (156, 157) that occurs in various types of parenchymal liver disease. However, Baker *et al.* (158) found that patients with liver disease excreted a very small proportion of injected vitamin B_{12}. Increased binding by serum protein may be the cause. Serum zinc is lowered in cirrhosis (155).

Serum proteins in liver disease.—Martin & Davis (162) found a gross increase in circulating globulin in patients with chronic liver damage. Transfusion of albumin had no effect on the circulating globulin of such patients. A thorough electrophoretic study of serum proteins in hepatobiliary diseases was made by Owen & Robertson (163). Elevated γ-globulin in hepatitis or cirrhosis and elevated α_2-globulin in neoplastic disease was helpful in recognition of these conditions although the information often was more easily provided by less elaborate methods. Distortions of the α_2-globulin zones in viral hepatitis were observed (164, 165); an extra component was often present (165). Schaffner *et al.* (166) found albumin concentrations to be closely correlated with hepatocellular function, and those of γ-globulin, especially γ_1, with hepatic inflammation. In liver disease, the polysaccharide associated with rapidly migrating proteins decreased while that of slowly migrating proteins increased. Aly (167) identified a rapidly moving pre-albumin component in serum similar to that in cerebrospinal fluid, and found it to be decreased in viral hepatitis. The advantages of pseudo-cholinesterase measurements for diagnosis of portal hypertension were upheld by Hunt & Lehmann (168). A scheme for fractional analysis of serum globulins was described by Greenspan (169) and applied to the differential diagnosis of jaundice.

Franklin & Kunkel (170) separated serum γ-globulin by electrophoresis on starch gels into three to ten subfractions and studied these with thymol-barbital, cephalin-cholesterol, and zinc-barbital reagents. The more basic (slowly migrating) components and certain basic proteins such as lysozyme, ribonuclease, and trypsin reacted with the thymol and cephalin-cholesterol reagents much more vigorously than did the more acidic γ-globulin. The latter reacted preferentially with zinc ions in solutions of low ionic strength. Napier (171) described a cupric ferricyanide reagent for evaluation of serum protein alterations in liver disease which correlated closely with the thymol turbidity test, but could be more easily prepared.

Profound alterations in the properties of the lipoproteins of serum were found in biliary cirrhosis by Russ *et al.* (172). The α- and β-lipoproteins normally present were almost entirely replaced by two or, at times, three ab-

normal lipoproteins, with lowered ratios of peptide to lipide and cholesterol phospholipide. Sedimentation constants were predominantly in the S_f 10 to 20 range. Similar studies in other types of hepatobiliary disease were described by Eder (173).

Fibrinolytic activity of serum in cirrhosis increased to six to eight times the maximal normal [Kwaan *et al.* (174)]. However, Fearnley (175) points out that the great lability of the fibrinolytic factors of serum have interfered with the application of such studies; immediate chilling and low temperature techniques are obligatory.

Metabolic abnormalities in liver disease.—Marked impairment of ketogenesis occurred in liver slices of rats with ethionine-induced liver damage as well as in intact rats, and in patients with cirrhosis [Recant (160)]. Fischer & Recant (161) found defective acetate metabolism and ketogenesis in mitochondria to be the cause.

Dihydroxy bile acids increased in the serum of patients with severe liver disease as much as fivefold, while little change occurred in cholic acid. Both types of bile acids increased in biliary obstruction [Carey (159)].

Plasma Proteins and Disease

Analbuminemia.—Bennhold, Peters & Roth (176) discovered a 31-year-old woman in whose serum no albumin could be detected either by electrophoresis or by immunochemical methods. The relatively trivial clinical symptoms included slight edema of the fingers, hypotension, and high erthrocyte sedimentation rate. The total protein (4.6 gm. per 100 ml.) consisted of: α_1, 7.4; α_2, 19.7; β, 32.1; and γ-globulin, 40.8 per cent of total protein. Colloidal osmotic pressure was about 58 per cent of the usual normal value. The serum of a brother also lacked albumin, but both parents had normal electrophoretic patterns. Two siblings had died.

Absence of beta globulin.—Riegel & Thomas (177) failed to find β-globulin in the serum of an 85-year-old woman two days before death. Iron binding capacity was nil, and serum Fe only 75 μg. per 100 ml. despite marked hemosiderosis.

Agammaglobulinemia.—Immunological and electrophoretic studies of sera from patients with congenital or acquired forms of agammaglobulinemia by Gitlin *et al.* (178) showed that at least two proteins, in addition to γ-globulin, were absent. Both migrated as β-globulins in agar gels. No deficiency of clotting factors could be demonstrated (179). Martin *et al.* (180) traced the development of hypogammaglobulinemia over a period of years in a patient with normal γ-globulin at the start of the illness. A survey of agammaglobulinemia has appeared (181).

Miscellaneous defects.—A case of afibrinogenemia, lacking factor V and a antihemophilic globulin, and accompanied by generalized cystine crystal deposition in the cells of the reticulo-endothelial organs, together with aminoaciduria, diabetes insipidus, and renal tubular insufficiency was described by

Burgstedt & Marx (182). Ulstrom *et al.* (183) described a transitory hypoproteinemia with absence of γ-globulin in infants.

Cholinesterase.—Low pseudocholinesterase activity in serum was encountered in two families by Lehmann & Ryan (184) and in a third by Allott & Thompson (185). The use of drugs or anesthetic agents with anticholinesterase activity is hazardous in such persons. High pseudocholinesterase activities in serum occur in the obese (186).

Macroglobulins.—Kratochvil & Deutsch (187) studied a crystalline preparation of macroglobulin. Although electrophoretically homogeneous, it included three major components, sedimenting at 18.1, 21.2, and 26.4 S, when studied in the ultracentrifuge; its antibody reacted strongly with normal human serum. Wallenius *et al.* (188) reported that as much as 4 per cent of the protein of normal serum behaved as macroglobulin in the ultracentrifuge. This normal macroglobulim contained five times as much carbohydrate as was found in γ-globulin having a sedimentation constant of 7 S (192, 193) but resembled in composition the α_2-globulin of 19 S. However, additional immunochemical evidence was presented to support the view that in macroglobulinemia the macroglobulins are abnormal proteins (189, 190, 191). Vogler *et al.* (194) have described a combined electrophoretic and diffusion method for identification of macroglobulins.

Cryoglobulins.—Putnam & Miyake (195) studied eight highly purified cryoglobulins. All except two differed from each other and from normal serum proteins in crystal form, solubility, isoelectric point, mobilities, sedimentation constant, ultracentrifugal or electrophoretic homogeneity, and N terminal amino acid residues. The differences were so extensive that the conclusion was drawn that cryoglobulins are truly abnormal proteins which are produced only in disease.

Myeloma proteins.—The abnormal proteins from four patients with multiple myeloma differed in isoelectric point and sedimentation pattern [Smith *et al.* (196)]. Although closely resembling γ-globulin in amino acid composition and molecular weight, they varied in their reactivity with antisera to human γ-globulins. One remarkable protein had a preponderance of basic amino acids, yet an isoelectric point of 4.7. It contained much carbohydrate. Korngold & Lipari (197) found that each of 24 multiple myeloma proteins cross-reacted with antiserum against normal γ-globulin, although the nature of the cross reactions suggested that myeloma proteins were altered proteins lacking the antigenic determinants which are present in normal γ-globulin. The carbohydrate content of myeloma proteins showed slight but significant differences from normal γ-globulins. Müller-Eberhard *et al.* (193) concluded that this finding could not be reconciled with the hypotheses that they are normal proteins produced in excess. Myeloma proteins of the β-type had more carbohydrate than those of the γ-type (193). An "α_2-" plasmacytoma was associated with hyperlipemia of extreme degree (198). Two myeloma proteins may occur in the same serum (198).

Studies of the antigenic properties of Bence-Jones proteins led Deutsch

et al. (199) to postulate that these may be portions of certain globulin molecules. Rapidly accelerated synthesis may exceed the capacity to carry out an essential step, e.g. the incorporation of methionine, in the normal mechanism of synthesis. Antigenic relationships between Bence-Jones and myeloma protein and normal γ-globulin were established by Korngold & Lipari (201). Putnam *et al.* (200) found that Bence-Jones proteins were excreted in the urine at a maximal rate many hours before the γ-globulin in plasma reached maximal activity. Administration of glutamic acid into which C^{14} was incorporated provided the labelling. The behavior of Bence-Jones proteins suggests that it is a precursor or abortive product of γ-globulin.

Amyloid disease.—Block *et al.* (202) and Rukavina *et al.* (203) studied 66 members of a family of which five had developed primary systemic amyloidosis. Twenty-nine had abnormal α_2-globulins, and the electrophoretic patterns of 15 showed an extra peak between α_1 and α_2. These 15 included the five with the disease. Abnormal lipoproteins in the S_f 20 to 25 (α_2) and 25 to 40 (β_1) classes were found in 27. An inherited aberration in lipoprotein metabolism was considered to be important in the pathogenesis of the disease. Letterer *et al.* (204) found that the amino acid composition of four preparations of amyloid varied. However, its composition differed substantially from that of liver protein and hyaline.

Miscellaneous studies of serum proteins.—Jayle *et al.* (205, 206) studied serum and urine proteins in the nephropathies, especially the α_2-mucoprotein, haptoglobulin. Studies of serum proteins were made in uremia (208), cardiac decompensation (209), arteriosclerosis (210), chest diseases (211), neoplastic diseases (207), chronic rheumatic diseases (212), and lupus erythematosis (213). Generalized giant follicular hyperplasia of lymph nodes and spleen (Brill-Symmer's disease) caused enormously increased β- and γ-globulin concentrations [(198); see also (214)].

The significance of changes in α-globulins was reviewed by Scheurlen (215). Roboz *et al.* (216) isolated and measured the glycoprotein of cerebrospinal fluid of patients with either multiple sclerosis, nondemyelinating disease, or brain tumor. The polysaccharide content of proteins in diabetes (217) and in various disease states was studied (218). Schmid & MacNair (219) correlated proteins of synovial fluid with those of serum. Smith (220) studied a heparin-precipitable fraction of plasma. It is thought to be a labile form of circulating fibrinogen which markedly increases in concentration as a result of inflammatory disease.

Hyperparathyroidism and Other Disturbances of Calcium and Phosphorus Metabolism

The inadequacy of measurements of serum calcium and phosphate concentrations for the diagnosis of hyperparathyroidism is well known. Chambers *et al.* (221) described three supplementary procedures: (*a*) tubular reabsorption of phosphate calculated as follows:

$$TRP = 1 - \frac{\text{Phosphate clearance}}{\text{Creatinine clearance}}$$

(*b*) phosphate deprivation: the phosphate excretion after three and six days on a diet low in phosphate; and (*c*) calcium tolerance: the response of serum and urine calcium to intravenous infusion of calcium. Both (*a*) and (*b*), particularly, clearly defined abnormalities associated with hyperparathyroidism. Nordin & Fraser (222) previously had used a standardized calcium infusion test as an aid to the study of osteomalacia. Patients with osteoporosis retained abnormally large amounts of infused calcium and this led Finlay *et al.* (223) to calculate the "Four hour skeletal retention" of calcium.

The hypercalcemia and hypercalciuria of sarcoidosis was corrected by treatment with cortisone (224, 225). The response is useful for differentiation of the hypercalcemia of sarcoidosis from that resulting from other causes. Henneman *et al.* (226) found that excessive calcium absorption from the gastrointestinal tract in sarcoidosis was an important cause of the hypercalcemia. They attributed this to endogenous vitamin-D intoxication, and in view of the tendency, especially noticeable in one patient, toward increased absorption of Na, K, Mg and N, postulated a generalized effect of vitamin D on intestinal absorption. Cortisone decreased calcium absorption. Sodium phytate had a similar effect and appeared to offer promise for the treatment of the hypercalcemia.

Idiopathic hypercalcemia of infancy may have grave effects and is being found with much greater frequency than in the past (227). Serum calcium, cholesterol, and urea are elevated (228). Calcium-free diets (229) and cortisone (230) lower the serum calcium. Balance studies show a large absorption of calcium from the gastrointestinal tract (227). Two types of the disease were identified (231); one with defective mentality and other congenital abnormalities had a long course and poor prognosis; the other was more amenable to treatment. Vitamin D was held responsible by Morgan *et al.* (227), either because of overdosage or hypersensitivity. Forfar *et al.* (231) believe the cause to be a disturbance of cholesterol metabolism with the production of derivatives having a hypercalcemic action.

Thyroid Disease

Michel (232) listed the reactions involved in the genesis of the thyroid hormones as: (*a*) trapping of iodide by the thyroid; (*b*) oxidation of iodide to iodine; (*c*) iodination of tyrosine (and histidine); (*d*) coupling of the mono- and diiodotyrosine to form triiodothyronine and thyroxine. These are incorporated into thyroglobulin which serves as a store of hormone. A cathepsin hydrolyzes thyroglobulin to liberate diffusible hormones. Thyroid deshalogenase deiodinates the several iodine compounds, especially mono- and diiodotyrosine, but not thyroxine, 3,5,3-triiodothyronine, or 3,3-diiodotyrosine. Ten to 20 per cent of the plasma iodine is present as inorganic iodide, 70 per cent as thyroxine, usually some as 3,5,3-triiodothyronine, with somewhat

less as 3,3-diiodotyrosine, and traces as 3,3,5-triiodothyronine. The liver rapidly concentrates all thyroid hormones and excretes these as glucuronic acid conjugates in bile. Deshalogenases exist in other tissues, notably the kidney.

Certain forms of cretinism have now been traced to specific defects in iodine metabolism. Stanbury & Hedge (254) several years ago described cretinism caused by the absence of an oxidase that converts iodide to iodine. A second defect, described in two sisters by Stanbury, Ohela & Pitt-Rivers (234), resulted from failure to couple iodotyrosines. Although uptake of iodine was high, thyroxine and triiodothyronine were found in very low concentrations in the thyroid gland. A clue to the cause of a third form of cretinism was provided by Costa *et al.* (235) with the discovery of diiodotyrosine in the blood of two cretins. Stanbury *et al.* (236) described three additional cretins with goiter and hypothyroidism which appeared to be caused by congenital lack of deiodinase. Mono- and diiodotyrosine, labeled with I^{131}, were found in blood and urine after oral administration. The defect was familial in occurrence; two of the three were brothers. The third had five relatives who were unable to deiodinate efficiently. McGirr *et al.* (237) provided conclusive evidence of the hereditary nature of this defect by studies of a family of itinerant tinkers with nonendemic goitrous cretinism. In one patient, 27 per cent of the plasma iodine and 60 per cent of the urinary iodine was diiodotyrosine. All told, ten members of the family showed abnormalities. The pedigree suggested that a single autosomal recessive gene was involved. A lack of deshalogenating enzyme was held responsible for the syndrome (Querido *et al.*, 238).

Thyroid tissue slices of a patient with familial goitrous cretinism failed to remove iodine from I^{131}-labeled diiodotyrosine, whereas sheep, bovine, and human thyroid tissue (from patients with Graves' Disease or nodular goiter) did so. A general somatic failure of the normal process of deshalogenation, leading to a life-long loss of thyroid hormone precursors in the form of mono- and diiodotyrosine from the body, thus was responsible for the hypothyroid state and hyperplastic goiter.

Losses of organic iodine in urine and feces in the nephrotic syndrome, together with failure of the pituitary-thyroid mechanism to respond adequately, may explain certain signs of hypothyroidism associated with nephrosis (239).

Cobalt was found to interfere with thyroid function and at times to produce myxedema (240, 241). According to Kirkwood & Fawcett (242), cobalt suppresses tyrosine iodinase in cell-free suspensions of thyroid tissue.

Serum butanol-extractable iodine often failed to increase in pregnancy terminated by spontaneous abortion (243), and the typical increase in percentage of thyroxine bound by thyroxine-binding protein of serum, which occurs in uncomplicated pregnancies, failed to occur (244). Although thyroxine in serum normally is found combined only with thyroxine-binding protein and albumin, a patient with carcinoma of the thyroid, metastasizing to

lymph nodes and lungs, showed extensive binding by γ-globulin (245).

The iodine-containing proteins of carcinomatous human thyroid tissue differed from the normal. The ratio of 3-iodotyrosine to 3,5-diiodotyrosine in the latter was 1:1 as compared with 5:1 in the former (246).

The thyrotrophic hormone activity of human serum, measured by the mouse assay method after being partially concentrated by precipitation with cold ethanol, was found elevated in thyrotoxicosis, normal or elevated in primary myxedema, and absent or low in pituitary myxedema (247). Findings by use of the chick assay method were similar (248). No correlation between thyrotrophic hormone activity in serum and exophthalmos could be demonstrated.

Fraser (249) has now published in full his appraisal of clinical tests of thyroid function including basal metabolic rate, uptake of I^{131}, and serum protein-bound iodine.

Triiodo- and tetraiodothyroacetic acid, although much less active than thyroxine in raising the basal metabolic rate in myxedema, lowered serum cholesterol and caused remission of clinical symptoms [Lerman & Pitt-Rivers (250)]. These substances also lowered serum cholesterol concentrations in some euthyroids without change in basal metabolic rate (251, 252).

Miscellaneous

Sarcoidosis.—Uncertainty as to whether sarcoidosis is a distinct disease or a granulomatous form of tuberculosis has long existed. Following the suggestion of Consden & Glynn (253), Nethercott & Strawbridge (145) detected in the lesions of sarcoid certain substances known to occur in bacteria but not in human tissues. α-ε-Diaminopimelic acid and mycolic acid were identified. Both are known to occur in *Mycobacterium tuberculosis* and related microorganisms.

Tuberculosis.—Crafton *et al.* (107) found that some patients with tuberculosis, while on antibacterial therapy developed critically low serum potassium concentrations. Unless recognized and treated, death resulted. A combination of low intake of potassium by the severely ill patients, mild diarrhea, and occasional vomiting was responsible.

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BIOCHEMISTRY IN THE U.S.S.R.[1]

By Jakob A. Stekol

The Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia, Pennsylvania

The articles reviewed here were read in the original language, and the review consists of a selection of the more important contributions by Soviet biochemists to animal biochemistry. All reports on biochemistry of plants and microorganisms, the effects of ionizing radiations on plants and animals, and on plant and animal nutrition were omitted because of the exigencies of space. Not all of the pertinent Soviet journals were available to the reviewer, and certain books, monographs, and pamphlets of biochemical interest could not be procured. The review, then, is not claimed to be complete. The backlog of Soviet biochemical literature, unfamiliar to the U. S. biochemists, is quite large. All the reviewer can do at this time is to present the gist of an accurate translation of articles, covering the past few years, selected and correlated to meet the stipulated requirements.

Pronin (1) has made an excellent suggestion that Soviet biochemists inaugurate reviews similar to the *Annual Review of Biochemistry* and *Advances in Enzymology* in which Soviet authors might systematically review their own progress. Such a review, translated into English, would be of help in situations where the Russian language is a serious barrier to exchange of biochemical information.

In view of the seriousness of charges made by the Editorial Board of *Biokhimiya* against the Coris, while defending the claims of Petrova, the work of Petrova and the Coris on the branching and debranching enzymes is reviewed here, accompanied by a communication from Dr. G. T. Cori in regard to these charges.

Carbohydrate metabolism.—Petrova (2) reported that the cleavage of carbohydrates in rabbit and rat muscle proceeds via phosphorolysis and hydrolysis. The amylase found in the muscle is activated by chlorides and, judging by various properties of the enzyme, it belongs to the alpha type. In another report, Petrova (3) observed that the maltose-splitting enzyme, maltase, is active in minced muscle and in extracts of skeletal muscle of adult rabbits, with an optimum pH of 5.6 to 6.8. Maltase is stable in acid solutions but, when the extracts are treated with alcohol, maltase activity is lost. This property was utilized in the preparation of maltase-free amylase. Dextrin, maltose, and glucose were found to be the products formed from starch and glycogen by the action of muscle α-amylase. In 1948 Petrova (4) reported on the discovery of a new muscle enzyme, called amylose isomerase, which

[1] The survey of the literature pertaining to this review was concluded in October, 1956.

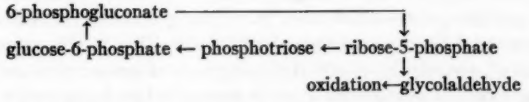
hydrolyzes and synthesizes polysaccharides with 1,6-linkages. According to Petrova, this enzyme is distinct from other known enzymes of muscle which act on polysaccharides with the α-glucoside linkage in the 1,4-position (phosphorylase, amylase, and maltase). The detailed procedure for the preparation of this enzyme is described. By the combined action of phosphorylase and amylose isomerase on glucose-1-phosphate, a polysaccharide akin to glycogen is formed. In another report, Petrova (5) described a method of separation of amylose isomerase from muscle and its chemical properties. She found that amylose isomerase is either identical with muscle globulin X or that the enzymatic activity of amylose isomerase is associated with globulin X and could not be separated from it. A more active preparation of the enzyme could be obtained, not by dialysis as previously described, but by diluting the KCl solution to 0.01–0.016 *M* and by adjusting the pH with acid to 5.3 to 5.5, the isoelectric point of globulin X. Glycogen could be completely transformed into the Cori ester by a mixture of phosphorylase and amylose isomerase. Contrary to Swanson & Cori (6), muscle phosphorylase alone could not synthesize the Cori ester.

In 1951, Cori & Larner (7) reported on the discovery of a new enzyme which cleaves the 1,6-linkages of polysaccharides, and named it amylo-1,6-glucosidase. The Editorial Board of *Biokhimiya* (8) published in the same year a bitter statement in which resentment was expressed against the apparent ignoring by foreign biochemists, particularly in the United States of the published work of Soviet biochemists. An article by Petrova (9) in the same issue of *Biokhimiya* disputes the priority of the discovery of amylo-1,6-glucosidase by Cori & Larner (7) on the basis of the identity of this enzyme and method of its preparation, with amylose isomerase described by Petrova some years earlier. Petrova (9) also critically refuted the claim by Cori & Larner (7) that the new enzyme (amylo-1,6-glucosidase or amylose isomerase) does not possess reversible properties. It should be noted that Petrova employed the preparation obtained on adjustment of the pH of the muscle extract to 5.5, while Cori & Larner (7) discarded the precipitate that was obtained on adjustment of the pH to 5.5. The preparations employed by Petrova and Cori & Larner (7) could not have been identical. For the record, it should be pointed out that as early as 1943, Cori & Cori (10) reported that muscle glycogen is synthesized by the action of two enzymes. One is the muscle phosphorylase which forms the unbranched 1,4-linked polysaccharide of the type of amylose. The other enzyme, prepared from heart and liver tissues, transforms the amylose to the branched 1,6-linked polysaccharide, glycogen. Khaikina & Goncharova (11) separated the two glycogen-synthesizing enzymes from brain tissue by fractional precipitation with ammonium sulfate, and Palladin & Khaikina (12) have employed these enzymes in the study of synthesis and degradation of glycogen in various sections of the brain and determined the glycogen content therein.

According to a personal communication from Dr. G. T. Cori,

Amylose isomerase (or IA), described by Petrova (4, 5) is presumed to have the property of synthesizing or breaking the 1:6 bonds at branch points of polysaccharides. According to Petrova, the enzyme acts on undegraded starch. It is very doubtful that such a reversibly acting enzyme exists in muscle, and we have never claimed priority for the discovery of such an enzyme. Our investigations have shown that two separate enzymes are involved. The branching enzyme (10) acts only in one direction and forms the 1:6 bonds from 1:4 bonds by transglucosidation of chains previously elongated by phosphorylase action, a mechanism which has been definitely established by Larner (13) with isotopically labeled glycogen. The debranching enzyme (7) acts only on limit dextrin formed by phosphorylase from glycogen or amylopectins, and the product of its action is free glucose which is formed by hydrolysis of the 1:6 bonds. The debranching enzyme does not act on undegraded glycogen, starch or amylopectins, and its action, being hydrolytic, is largely irreversible. Petrova's papers are confusing because of inadequacy of methods used for enzyme purification and lack of characterization of products formed. Even with her best enzyme preparation, incubation periods of 12 hours to 6 days were required to demonstrate changes in iodine color and reducing power. Even our early, crude preparations required only 15 to 60 min. of incubation at 30° for complete degradation of 12 mg. of glycogen. The fraction, which according to Petrova contains IA, is discarded in our preparations of brancher and debrancher enzyme. It should be also pointed out that the original article by Petrova [*Doklady Akad. Nauk S.S.S.R.*, **58,** 432 (1947)] was not abstracted in *Chemical Abstracts* until September 25, 1950 [*Chem. Abstr.*, **44,** 8393 (1950)], by which time our findings on brancher and debrancher had already appeared in print. One must deeply resent the statements made in *Biokhimiya* (8) that we were deliberately suppressing evidence known to us in order to claim priority.

Barkhash & Timofeeva (14) reported that the liver, kidney, and brain tissues of rats, or tissues of Egyptian beet, sugar beet, wheat, tea, navy bean, and potato shoots contain enzymatic systems which oxidatively degrade glucose-6-phosphate, 6-phosphogluconate, and ribose-5-phosphate, and which are similar to those previously described for yeast. 6-Phosphoglucono-dehydrase was purified from systems which further degrade pentose phosphate and from glucose-6-phosphate dehydrase. The enzymatic activity of 6-phospho-gluconodehydrase was raised thereby tenfold. Under the action of the purified preparation of this enzyme pentose accumulates in amounts which are almost equivalent to that of degraded 6-phosphogluconic acid. It has been further shown that the preparation from liver can cleave ribose-5-phosphate aerobically or anaerobically. A hypothetical scheme for this degradation was proposed:



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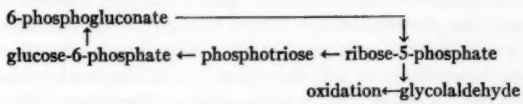
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pentosemonophosphate oxidation or "shunt," and fermentation or the Embden-Meyerhof route is synonymous with "dichotomous" oxidation (Engelhardt). In a later work, Barkhash & Timofeeva (15) report on the probable steps involved in the direct or apotomous oxidation of glucose, and data are provided for (*a*) the isomerization of ribose-5-phosphate to ribulose-5-phosphate; (*b*) transition of pentose phosphate to heptulose phosphate and a triose phosphate; and (*c*) further transformation of heptulose phosphate with the formation of hexosemonophosphate. The transformation of hexosemonophosphate from heptulose phosphate is proposed to take place via intramolecular transfer of the dihydroxy acetone portion of the heptulose to triosophosphate with the formation of fructose-6-phosphate (via aldol condensation) and erythrose-4-phosphate. The latter product could not as yet be identified. Similar views were expressed by Horecker & Symrniotis (16) in their work on transaldolase. The peculiarity of the transketolase reaction (and that of transaldolase) is that one of its participants, glycolaldehyde (in case of transketolase), is capable of reacting only in *statu nascendi*, i.e., at the moment of its cleavage from ribulose-5-phosphate (or hydroxypyruvate.) The two-carbon residue is then transferred to ribose-5-phosphate. The scheme offered by the authors (15) illustrates the possible participation of transketolase in the formation of active glycolaldehyde and its reaction with an aldehyde acceptor:

$$CH_2OH \cdot CO \cdot R_1H + \text{Transketolase} \rightarrow CH_2OH \cdot COH\text{-Transketolase} + R_1$$
$$CH_2OH \cdot COH\text{-Transketolase} + CHO \cdot R_2 \rightarrow CH_2OH \cdot CO \cdot CHOH \cdot R_2 + \text{Transketolase}$$

(Thiamine pyrophosphate is an essential cofactor.)

The questions of specificity of transketolase and of the concrete role of thiamine pyrophosphate, its co-factor, require, however, further study. The authors (15) once again emphasize the importance of this apotomous route of glucose oxidation (pentosemonophosphate route) versus the glycolytic or Embden-Meyerhof route, indicating that as far back as 1938 Engelhardt & Barkhash (17) stressed the fundamental importance of this route as the site of application of the Pasteur reaction. Recent observations of Bloom & Stetten (18), which indicated that as much as 50 per cent of the catabolyzed glucose in liver proceeded via the apotomous route, are cited in support of these views.

El'tsina (19) observed that in malignant ascites cells phosphorylation coupled with glycolysis is much more extensive than phosphorylation coupled with respiration, the ratio being seven to three. The energy of glycolysis and respiration in malignant cells is directly utilized for synthetic purposes, and the processes of respiration and glycolysis furnish at the same time the material for the synthesis. In 1944 Engelhardt (20) pointed out the importance of a study in which the energetics of respiration and glycolysis in malignant cells is considered as an integral and an inseparable part of the process of synthesis of the living cell and of its component parts. Aerobic glycolysis of malignant cells, discovered by Warburg 30 years ago, is still the

most outstanding characteristic of malignant cells. It has been tacitly assumed that aerobic and anaerobic glycolysis are chemically identical processes; that malignant cells simply retained the process that existed in normal cells under anaerobic conditions with a difference that it functions in malignant cells under aerobic conditions. Engelhardt (21) questioned the chemical and qualitative identity of anaerobic and aerobic glycolysis. He presented data, obtained by his collaborator, El'tsina, which demonstrated that the exclusion of either aerobic glycolysis or respiration by bromoacetate or NaCN, respectively in malignant ascites cells resulted in inhibition of incorporation of C^{14} of uniformly labeled glucose into proteins of the cells. Exclusion of glycolysis did not inhibit respiration, and the exclusion of respiration increased glycolysis, as expected. Since the incorporation of C^{14} of the uniformly labeled glucose into proteins was inhibited when either respiration or glycolysis was excluded, the conclusion was drawn that neither respiration nor glycolysis separately is able to serve as a source of energy and of structural material for protein formation from glucose. This can occur only when both processes are allowed to proceed simultaneously. From these observations Engelhardt (21) makes the proposal that glycolysis under aerobic conditions, in conjunction with respiration as it exists in malignant cells, is not aerobic glycolysis, that is, glycolysis which is able to function under aerobic conditions, and that anaerobic glycolysis and aerobic glycolysis are not qualitatively identical processes. This idea will undoubtedly stimulate further work, and Engelhardt (21) suggests a few problems in this direction using glucose-1-C^{14}, including the possibility that lactic acid in tumors originates via the apotomous (respiration, pentose-monophosphate route) of glucose degradation and not exclusively via the direct split to trioses (dichotomous route, fermentation, or Embden-Meyerhof route). The possible intermediates of such an apotomous route of glucose degradation, which may give rise to lactic acid, have been presented by Barkhash (22).

El'tsina (23) further showed that the exclusion of respiration in malignant ascites cells, while it increased the extent of glycolysis, did not affect the level of phosphorylation. Exclusion of glycolysis, on the other hand, inhibited phosphorylation in malignant cells by 40 per cent. She (23) presented further data to show that the inhibition of glycolysis or respiration in malignant cells inhibited the incorporation of C^{14} of uniformly labeled glucose into nucleic acid, lipids, and proteins. She suggested that the fact that glucose carbon incorporation into nucleic acids was inhibited in the presence of respiration indicates that the synthesis of the pentose moiety of the nucleic acids proceeds via the condensation of 3- and 2-carbon residues of glucose and not via the direct oxidation of glucose-6-phosphate. The author further suggested that consideration of the significance of aerobic glycolysis in malignant cells exclusively from the energy standpoint constitutes an oversimplification. The importance of aerobic glycolysis, being a characteristic feature of malignant cells, lies in its functional significance to the metab-

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olism of the cell. Lisovskaya (24) showed that the extent of incorporation of inorganic P_{32} into the phosphoproteins fraction of the proteins of rat brain (grey matter) is intimately related to oxidative processes. Factors which inhibited respiration inhibited the incorporation of P_{32}. Inhibition of oxidative phosphorylation similarly inhibited the incorporation of P_{32} into phosphoproteins.

Engelhardt & Lisovskaya (25), using P_{32}, studied the effect of various substrates of oxidation on phosphoprotein synthesis, and the role of ATP in this process. It was found that the incorporation of P_{32} occurred only in the presence of ATP or during its active synthesis. Brain slices oxidized fructose as energetically as glucose. Four groups of substrates were investigated in relation to phosphoprotein synthesis: (*a*) glucose and mannose were oxidized by brain slices, generated ATP, and accelerated phosphoprotein synthesis; (*b*) fructose and pyruvic acid were oxidized by brain slices, generated ATP, but did not stimulate phosphoprotein synthesis; (*c*) glutamic acid and succinic acid were oxidized by brain slices, did not generate ATP, or stimulate phosphoprotein synthesis; and (*d*) galactose, fumaric, and malic acids were not oxidized by brain slices and they did not stimulate phosphoprotein synthesis. At present it is not clear why fructose and pyruvic acid which are extensively oxidized by brain slices and which generate ATP do not accelerate phosphoprotein synthesis. Apparently the presence of ATP is an essential but not sufficient condition for the synthesis of phosphoproteins in the brain. While this work was in progress, Findlay *et al.* (26) reported similar results obtained on cat brain slices.

Lisovskaya (27) continued these studies with the thought that the process of glycolysis could serve as an energy generator for the synthesis of phosphoproteins in the brain. Of particular interest was fructose which, as has been shown above, underwent extensive oxidation in the brain, was capable of regenerating ATP, and did not affect phosphoprotein synthesis. The reasons for the different metabolic behavior of glucose and fructose in the brain were therefore investigated. It was found that the extent of "fructolysis" (lactic acid formation from fructose) under aerobic conditions was 27 per cent of the aerobic glycolysis (glucose substrate) and only 8 per cent of the anaerobic glycolysis of glucose. This confirms the findings of Loebel (28) and Meyerhof & Lohman (29). Resynthesis of ATP with fructose as the substrate under anaerobic conditions is also low, being about 15 to 40 per cent of that observed with glucose as the substrate under similar conditions. For these reasons considerations of only the energy of oxidation of substrates in the question of protein synthesis is insufficient, simply because the oxidation level of fructose and glucose by the brain is the same, yet fructose is ineffective in the synthesis of protein in the brain. Low glycolysis of fructose must, therefore, be a significant factor in the poor synthesis of protein in the brain with fructose as the substrate. Since hexokinase in the brain is a system common to fructose and glucose, and since phosphorylation of fructose is about 1/50 that of glucose (30), it follows that the low rate of

phosphorylation of fructose leads to a low rate of glycolysis of fructose. Rapid oxidation of fructose by brain tissue must, therefore, mean that fructose bypasses that reaction which under anaerobic conditions is the "bottleneck." According to Meyerhof (31), in this case the "bottleneck" is the hexokinase reaction. However, phosphorylation of sugars by hexokinase is the first step in degradation of hexoses, common to oxidative and non-oxidative paths. If, during the oxidation of fructose, this were the essential step, then the oxidation of fructose could not have proceeded as fast as that of glucose. Conversely, if the oxidation of fructose and glucose is the same, then oxidation of fructose cannot possibly go via the fructose-6-phosphate step, but must go by a different path. In other words, glycolysis and oxidation of fructose by brain tissue from the start proceed via different paths. The oxidation of fructose in the brain cannot be the terminal step of glycolysis, neither can it be that of apotomous or stepwise degradation, if in both cases the first reaction is the hexokinase reaction. The author admits, however, that from a single observation a rather general deduction which excludes the hexokinase reaction has been made concerning the oxidative path of fructose in the brain. Further data are necessary for a more direct demonstration of this view.

Vladimirov *et al.* (32) examined phosphoprotein synthesis in rat brain by means of P^{32} and found that the determination of phosphoprotein by the method of Schmidt & Thannhauser (33), followed by the precipitation of inorganic phosphate according to Delory (34) after hydrolysis, results in too high a value for its specific activity. A more correct value for the specific activity is obtained by extraction of the phosphomolybdic blue with isobutanol. The true specific activity is thereby reduced to about one-half, and this value closely corresponds to the specific activity of phosphoserine which was isolated from phosphoproteins. It would seem that other P_{32} bonds exist in the phosphoproteins which may affect the apparent specific activity of phosphoproteins isolated after P^{32} administration.

Etinhoff & Gershenson (35) found that the inhibition of respiration under aerobic glycolysis is preserved during various periods (seven to ten days) of development of mouse ascites cells. Utilization of oxygen is increased, after the addition to the system of methylene blue, without alteration in the extent of glycolysis. The amount of reducing substances in the medium decreased. Thus, methylene blue creates conditions for parallel existence of glycolysis and aerobic oxidation. Ethylurethan decreased the respiration but not glycolysis and abolished, in part, the reverse Pasteur effect. Compounds containing the thiol group (cysteine, dimercaptopropanol) completely abolished the reverse Pasteur effect, and restored respiration to the level obtained in the absence of glucose. Increased oxygen consumption is explainable on the basis of mobilization of endogenous sources in the cell, since no reduction in the amounts of reducing substances occurs under these conditions.

Epelbaum & Kantor (36) observed that denervation of mouse muscle

leads to lowering of the glycolytic activity in the muscle. After 30 to 35 days following the ligation of sciatic nerve the muscle practically lost the ability to convert glucose to lactic acid. After 20 to 25 days following the denervation, aldolase activity of the muscle was lowered by 73 to 89 per cent. Tendotomy (ligation of Achilles tendon) led to large losses of muscle tissue, but aldolase activity remained unaltered.

Seitz & Engelhardt (37) observed that sodium azide and dinitrophenol, poisons which inhibit coupled phosphorylation, also inhibited the Pasteur effect. Erythrocytes of pigeons and geese, baker's yeast, and malignant ascites cells were used. In the ascites cells, dinitrophenol sharply increased glycolysis and inhibited coupled phosphorylation, but left respiration unaffected. In normal cells, the poisons which inhibited coupled phosphorylation also inhibited the Pasteur effect. The conclusion was drawn that the two processes are interrelated and that the observed effects are induced through steps which are common to both processes. Seitz & El'tsina (38) reported on the isolation of the anti-Pasteur effect factor from various normal animal and plant tissues and from malignant neoplasms. The action of this factor was similar to azide, dinitrophenol, etc., i.e. it induced aerobic glycolysis without inhibiting respiration, and at the same time it uncoupled phosphorylation. Chemical evidence presented by the authors pointed to a steroid nature for this "anti-Pasteur effect factor."

El'tsina (39) further reported that the addition of azide to malignant ascites cells increased the extent of incorporation of C^{14} of uniformly labeled glucose into proteins, as well as the extent of glycolysis, as has been reported previously by Seitz & Engelhardt (37). Thus, azide in increasing glycolysis and in uncoupling phosphorylation from respiration leaves unaffected one of the most important functions of respiration, namely, the transport of hydrogen via the system of intermediates from the substrate which is being oxidized. It is clear that in cells exposed to azide and to increased glycolysis there exists a respiratory process which participates in the terminal stages of oxidation of glucose. Under such conditions the assimilation of glucose for protein synthesis in malignant cells increases. The uncoupling of aerobic phosphorylation by azide is apparently insufficient to inhibit protein synthesis from glucose in malignant cells, because glycolytic esterification of phosphorus is increased by azide. For this reason, such uncoupling agents as azide cannot be considered satisfactory inhibitors of malignant growth. Rosenfeld, Shubina & Kuznetsov (40) reported that a dextran-cleaving enzyme exists in beef and rabbit spleen which, in acetate buffer under sterile conditions, cleaves dextran. Liver, muscle, whole blood, plasma, or kidney extracts were inactive under similar conditions.

Lukomskaya (41) reported that preparations from liver, muscle, and spleen (rabbit) contain an enzyme which cleaves isomaltose. This enzyme resembles oligo-1,6-glucosidase found in mucous membrane of intestine (42), but it is different from R-enzyme and amylose isomerase or amylo-1,6-glucosidase which does not cleave isomaltose.

Kainova & Petrova (43) synthesized a series of glycogens *in vitro* from glucose-1-phosphate by the use of phosphorylase and amylose isomerase. The molecular weight of the glycogens approached that of natural glycogens. The synthetic and natural glycogens differed from amylopectins in their reaction to iodine. The synthetic and natural glycogens formed complexes with myosin, which were demonstrated by shifts in the ultraviolet absorption maximum. Stepanenko, Khlurova, and Zubrilina (44) and Stepanenko (45), employing $NaHC^{14}O_3$, found that epinephrine stimulated the synthesis of glucose residues of the glycogen chain in normal starving rats. Epinephrine was ineffective in alloxan-diabetic starving rats. It was also found that the exchange reactions occur much more slowly in the central branched portions of glycogen than in the periphery of the molecule. The authors suggest that a new characteristic of diabetes consists in the alteration of the synthesis of glucose residues of glycogen. According to Chernyak (46), hemolyzed human erythrocytes form 2,3-diphosphoglyceric acid from 3-phosphoglyceric acid in the presence of cozymase and inorganic phosphate and in the absence of ATP. The intermediate in this reaction is phosphoglyceraldehyde. Ginodman (47) employed the tracer technique in determining the equilibrium constant of hydrolysis for glycerophosphate and glucose-6-phosphate. K_e for glycerophosphate at pH 7 and 25° was 0.63, and for glucose-6-phosphate, under the same conditions, K_e was 1.48. The calculated ΔF for glycerophosphate was -2100 cal./mole, and for glucose-6-phosphate the ΔF was -2600 cal./mole. Under physiological conditions, i.e., glucose at a level of 90 mg. per cent phosphate 0.005 M, and glucose-6-phosphate 0.005 M, the ΔF is -5700 cal./mole. Thus, this "energy-poor" phosphate bond during hydrolysis under physiological conditions yields a sizable amount of energy.

Severin *et al.* (48) claimed that carnosine and anserine participate in glycolytic reactions coupled with phosphorylation, and Severin *et al.* (49) further indicated that carnosine and anserine are involved in oxidative phosphorylation as well. Mishukova (50) found that the transformations of carbohydrates in homogenates and extracts of muscle of Prussian carp follow the same path as in similar preparations of other vertebrates, but at a slower rate and predominantly under anaerobic conditions. Carnosine and histidine in the concentration of 100 mg. per cent accelerated glycolytic oxidation of glucose but inhibited dephosphorylation of phosphopyruvate. Dikanova (51) studied the formation of phosphoglyceric acid from fructose diphosphate by homogenates of smooth muscle of chickens or pigeons. She found that carnosine had no effect on the binding of inorganic phosphate, and that, in the presence of fluoride, carnosine and creatine had no effect on the formation of labile phosphate. The conclusion was that carnosine does not participate in the formation or accumulation of energy-rich phosphate compounds.

Venkstern & Engelhardt (52) presented data for demonstration of the activity of adenosinetriphosphatase located on the surface of the nucleated

red blood cells. The adenosinetriphosphatase activity of intact nucleated erythrocytes is small compared to that of the muscle. The activity is greatly increased by destruction of the cell structure (53). In this work the name of ecto-adenosinetriphosphatase is proposed for the adenosinetriphosphatase which is located on the surface of the nucleated erythrocytes and such cells as malignant ascites cells (54) and yeast (55). Nonnucleated erythrocytes had no ecto-adenosinetriphosphatase activity. Ninety to 95 per cent of the entire adenosinetriphosphatase activity of nucleated erythrocytes is due to ecto-adenosinetriphosphatase of the intact cell. The measurements of ecto- and endo-adenosinetriphosphatase activities of the same nucleated erythrocytes was accomplished by inhibiting respiration and resynthesis of ATP by NaCN. Ecto-adenosinetriphosphatase cleaves both labile phosphates with equal ease. Adenylic acid phosphate is not affected. The ecto enzyme is relatively thermostable: heating at 50 degrees for 20 minutes did not affect its activity. It is activated by Mg^{++} and is inhibited by Ca^{++} and fluoride (at 1×10^{-3} *M*, inhibition is 30 to 40 per cent). Monoiodoacetate in a concentration of 0.01 *M* has no effect. Alpha-2,4-dinitrophenol, in a concentration of 0.001 *M* inhibits, and in lower concentrations, activates it. Neither ATP nor adenylic acid penetrate the cell wall of nucleated erythrocytes, and the adenosinetriphosphatase activity of the intact cells cannot be explained on the basis of penetration of ATP inside the cell, cleavage by the endo enzyme within the cell, and release of the free phosphate into the surrounding medium. One of the possible functions of the ecto enzyme, as suggested by the authors, could be regulation of the ATP concentration in the surrounding medium. Other, more significant functions, are as yet unknown.

Shabanova (56) studied metabolic changes in muscle of rats in which muscular dystrophy was induced by deficiency in vitamin E. The content of acid-soluble organic phosphorus, particularly of the easily hydrolyzable fraction to which ATP belongs, was decreased by 50 per cent in the muscle of dystrophic rats. Creatine, carnosine, and particularly anserine of the muscle also decreased. Amino nitrogen content was increased. The oxidation of phosphoglyceraldehyde was reduced. The accelerating effect of carnosine on the rate of binding of inorganic phosphate and formation of phosphoglyceric acid, observed in normal rat muscle, was absent from the muscle of dystrophic animals.

Severin, Meshkova & Rasumovskaya (57) reported that the addition of cyanide to pigeon breast muscle homogenates in phosphate buffer did not affect the P/O ratio. Addition of anserine in the presence of cyanide to the homogenates increased the P/O ratio; carnosine was ineffective. Azide uncoupled oxidative phosphorylation, and the addition of anserine completely counteracted the effects of azide on coupled phosphorylation. Phosphorylation of creatine was increased by the presence of azide and anserine in the medium. Carnosine and histidine were less effective than anserine in coun-

teracting the effects of azide; β-alanine was ineffective. However, anserine was ineffective in restoring coupled phosphorylation which was inhibited by dinitrophenol. These differences in the action of anserine in restoring coupled phosphorylation inhibited by azide or dinitrophenol indicate that the two uncoupling agents do not act via the same mechanisms.

Petrova (58) found that in extracts of normal rabbit liver the degradation of glycogen proceeds by way of hydrolysis and phosphorolysis, and the rate of phosphorylytic cleavage is increased and that of hydrolytic cleavage is decreased in alloxan-diabetic animals. In normal animals the synthetic capacity of phosphorylase in liver extracts is weak, but the activity of phosphoglucomutase is considerable. In alloxan-diabetic animals the synthetic capacity of phosphorylase in similar extracts is increased, and the activity of phosphoglucomutase is decreased. Tseitlin (59) found that the addition of chloral hydrate or medinal to homogenates of rabbit heart muscle decreased the binding of inorganic phosphate, phosphorylation of creatine, and the formation of fructose diphosphate. This effect was observed only under anaerobic conditions. The synthesis of ATP was also inhibited by the drugs. While these sedatives apparently affect the activities of enzymes of oxidative phosphorylation, they are without any measurable effect on the activities of adenosinetriphosphatase and phosphopherases.

Leites (60) described a method of preparation of extracts of pancreas which contain lipocaic. In contrast to the preparation of Dragstedt, the Leites preparation was free of glucagon. An attempt to settle the following questions is described: (*a*) Is lipocaic a product of internal or external secretion of the pancreas, what morphological substrate is concerned with its formation, and what are the paths of the nervous regulation of its secretion; (*b*) what is the relationship of lipocaic to the lipotropic food factors, such as choline, methionine, and inositol; and (*c*) what is the mechanism of action of lipocaic? Alloxan diabetes in dogs does not produce fatty livers or ketonuria. Removal of the pancreas from alloxan-diabetic dogs resulted in ketonuria and fatty livers without an increase in hyperglycemia. Orally administered lipocaic preparation to depancreatized alloxan-diabetic dogs reduced ketonuria and removed fat from the liver. Ligation of the ducts of external secretion of the pancreas of dogs and rabbits did not result in production of fatty livers or ketonuria in five to seven months, although atrophy of ascinar tissue was observed. However, fatty livers and ketonuria were developed in 7 to 30 days in depancreatized dogs. Therefore, lipocaic production is not concerned with the external secretion of the pancreas, and the site of its formation is neither the ascinar tissue nor the beta cells. This is supported by the observation that lipocaic could be extracted from the pancreas of alloxan-diabetic dogs with totally atrophied ascinar tissue. Administration of alloxan and ligation of the ducts of external secretion in dogs produced not only atrophy of ascinar cells and necrosis of islet tissue, but also the degeneration of epithelium of small ducts from which arise the

alpha cells of the islets. It is proposed by the author that the site of lipocaic formation is the epithelium of small ducts and the alpha cells. After the ligation of wandering nerves under the diaphragm in dogs with ligated ducts of external secretion of the pancreas, fatty livers developed in 10 days, reaching a fat content of 46 per cent in 40 days. In these dogs no lipocaic could be extracted from the pancreas. The lipocaic preparation reduced the fat content of the livers of rats which were maintained on diets of 2.5 to 5 per cent casein. The preparation did not contain any casein-hydrolyzing enzymes. However, experiments are described which indicate that choline and methionine are required for the activation of lipocaic, but neither choline nor methionine alone, without lipocaic, was effective in phospholipide synthesis during acute fatty infiltration of the liver. Leites makes the suggestion that lipocaic stimulates the activity of lipotropic food factors in relation to their effect on phospholipid formation and the transfer of the latter from the liver. The mechanism of the lipotropic effect of lipocaic is thus concerned with the activation of phospholipid exchange (in the presence of the lipotropic food factors) and in the oxidation of fatty acids in the liver. Other possible paths of action of lipocaic are under study. This lipocaic preparation is at present in use clinically in the U.S.S.R. on diabetics with fatty livers and in patients with hepatitis.

Proteins and amino acids.—Orekhovich *et al.* (61) prepared alpha and beta crystallines from eye lens of cattle by means of electrophoresis and ammonium sulfate precipitation. Alpha crystalline is soluble in water, with an isoelectric point of pH 5.2 and mobility at pH 8, and ionic strength of 0.2, of -4.2×10^{-5} $cm^2/sec^{-1}/volt^{-1}$. The protein is very sensitive to denaturing agents. Beta crystalline is easily soluble in water, with an isoelectric point of 6.2 and mobility, at the same conditions as for alpha crystalline, of -1.5×10^{-5} $cm^2/sec^{-1}/volt^{-1}$. Beta crystalline is more stable to denaturing agents.

Orekhovich *et al.* (62) determined the molecular weight and the degree of asymmetry of procollagen. Employing several solvents and two methods, including measurements of physico-chemical constants by independent means, the authors established the molecular weight of native procollagen to be about 600,000. Assuming that the shape of the procollagen particle corresponds to an elongated ellipsoid, the axial ratio was calculated to be $b/a = 1/500$. The length of the particle is about 670 mμ and the diameter, 1.28 mμ.

Gaisinskaya *et al.* (63) determined the electrophoretic mobility of eight protein components of dog blood serum: globulins ($alpha_1$, $alpha_2$, $alpha_3$, $alpha_4$, $beta_1$ and $beta_2$), albumin, and gamma globulin. $Alpha_3$, $alpha_4$ and $beta_1$ globulins were shown to be electrophoretically heterogeneous. The relative concentrations of albumin and of the three groups of globulins were determined in the sera of dogs which were kept for two to three months on a protein-free regimen. The resulting hypoproteinemia was mainly due to a

decrease in albumin without any decrease in the globulins. Parenteral administration of a preparation of serum (nonanaphylactic serum, prepared by V. N. Orekhovich) restored the albumin levels to normal in 12 to 17 days.

Kretovich *et al.* (64) prepared legumelin, vicilin, and legumin by the well-known methods of T. B. Osborne and by another method developed by the authors in which denaturation of the proteins by organic solvents was avoided, mainly by drying the proteins after dialysis *in vacuo* at low temperatures. Whereas proteins prepared by the Osborne methods were free of enzymatic activities, the same preparations isolated by the authors' method had considerable activities of catalase, peroxidase, invertase, dipeptidase, and carboxylase. Electrophoretic studies showed that the albumin of peas, legumelin, is a complex of four proteins which possess high enzymatic activities. The globulins, vicilin and legumin, behaved electrophoretically like single proteins, although they too possessed several different enzymatic activities. The authors somewhat belabor the point by insisting that so-called "reserve proteins" cannot be called "inert proteins," and that this erroneous notion should be removed from the texts.

Demin (65) reported a study of the effects of acetylcholine in relation to certain properties of proteins. Acetylcholine did not affect the viscosity or sorption properties of a series of solutions of proteins obtained from various tissues. Acetylcholine significantly increased, in the majority of cases, the concentration of titratable thiol groups of various tissue extracts and suspensions, without denaturing the proteins. The increase in titratable thiol groups, induced by acetylcholine, was attributed to increased reactivity of thiol groups. The mechanism of such increased reactivity under acetylcholine influence may be analogous to that proposed for a similar effect induced by urea (66), viz. that urea weakens the sulfur-hydrogen bond, thereby increasing its oxidizability. Local alteration in the distribution of charges and hydrogen bonds at a protein site to which the acetylcholine molecule becomes attached (formation of acetylcholine-protein complex) may lead to a reorganization of bonds and charges in more distant portions of the protein, thus favoring the "liberation" of thiol groups. Attention is called to the fact that acetylcholine favors increased oxidation of thiol groups of proteins by ferricyanide, and lowers the activity of thiol groups towards $HgCl_2$ (mercaptide formation). Acetylcholine apparently competes with $HgCl_2$ for the thiol groups of proteins.

Turpaev (67) investigated the significance of thiol groups in acetylcholine action on heart muscle. The muscle of frog heart, treated with $HgCl_2$, retains its contractile properties, but it loses the ability to respond to the inhibitory effects of acetylcholine. Addition of cysteine restores this ability. Loss of sensitivity of myocardium to acetylcholine occurs when about 6 per cent of tissue thiol groups are blocked by Hg^{++}. Using $Hg^{203}Cl_2$, the author has shown that only one-fifth of the accumulated Hg^{203} reacts directly with tissue structures which are responsible for the acetylcholine effect. During

the accumulation of Hg^{203}, in amounts of 0.1 to 0.15 mg. Hg per gram of tissue, an alteration in the sensitivity of myocardium to acetylcholine sets in, and this corresponds to 1.3 to 2 per cent of all the free thiol groups blocked by Hg^{203}. Simultaneous treatment of myocardium with Hg^{203} and acetylcholine sharply reduced the rate of accumulation of Hg^{203} in the tissue and lowered the rate of block of the sensitivity of the muscle to acetylcholine. These observations confirm the competitive action of acetylcholine and Hg^{++} for thiol groups of the muscle, and indicate the existence of probably an enzymatic mechanism, dependent on thiol groups, which is connected with the inhibitory action of acetylcholine on myocardium activity. These concepts are in accord with the enzymochemical theory of nerve excitation and inhibition developed by Koshtoyanz (68).

Yoffe (69) described selective reagents for the isolation, by paper chromatography, of tyrosine-containing peptides from incompletely hydrolyzed silk fibroin of *Bombyx mori*. Of several compounds synthesized, *m*-aminophenyl-trimethylammonium dihydrochloride and *p*-aminophenyl-trimethylammonium dihydrochloride proved to be most sensitive towards diazotization with tyrosine and precipitation of the dye with alkaloidal reagents. Employing these reagents, Yoffe (70) isolated from the incompletely hydrolyzed silk fibroin a large peptide containing tyrosine. After removal of the reagent, the isolated peptide was cleaved to several peptides and, simultaneously, tyrosine was converted to aminotyrosine, cleaved from the chain, and removed. The mixture of peptides was then chromatogrammed by two-dimensional paper chromatography, and 16 peptides were isolated. The structures of these peptides was then established by the method of Consden *et al.* (71). Later, Yoffe (72) synthesized three peptides which he has previously shown to be present among the 16 peptides isolated (70). The synthetic Ala.Gly., Gly.Ala.Gly., and Ala.Gly.Ala.Gly. were identical with the corresponding peptides that were isolated from the incompletely hydrolyzed silk fibroin. Ala.Gly.Ala.Gly. accounted for two-thirds of all the amino acid residues of silk fibroin of *Bombyx mori*. Neither Gly.Gly. nor Ala.Ala. was detected by Yoffe (70), but their possible existence was not ruled out. Ala.Tyr. and Tyr.Ala. were also absent (70). The conditions of partial hydrolysis of silk fibroin employed by Yoffe (70) were as follows: 10 gm. of silk fibroin in 100 ml. of 10.3 *N* HCl were allowed to digest for 1 hr. at 27°, then at 0 to 5° for 23 hr.

Kay *et al.* (73) used *Tussah* silk fibroin and hydrolyzed 1.07 gm. in 10 ml. of concentrated HCl for 48 hr. at 37°. By column chromatography they were able to detect Gly.Gly. and Ala.Ala., but not Ala.Tyr. or Tyr.Ala. or Ala.Gly.Ala.Gly. The isolated peptides accounted for about 90 per cent of the silk fibroin used. In the insoluble portion, on hydrolysis, alanine, glycine, serine and asparagine were present.

Orekhovich *et al.* (74) found that the reported incorporation of radio amino acids into plasma proteins and other proteins in phosphate buffer

at pH 7.5 (75) was due to contamination by bacterial proteins. Krizman *et al.* (76) dispute the validity of the conclusions by Orekhovich *et al.* (74) that bacterial contamination of proteins accounted for the incorporation of radio amino acids into various proteins within 2 hr. at 37° incubation. Under these conditions, according to the authors, the incorporation of radio amino acids into proteins isolated from rat liver is more intensive than the incorporation into analogous proteins isolated from rabbit liver. To top these results, the authors claim that incubation of various proteins with radio glycine at 100°C. also results in incorporation of the amino acid, and the pretreatment of proteins at this temperature lowers somewhat the extent of incorporation on subsequent incubation at 100°. Oxyquinoline, monoiodoacetate, and diphenylhydrazine slowed down the "incorporation" of radio amino acids into proteins *in vitro*, and the extent of this inhibition by the poisons was variable, depending on the temperature of incubation. Konikova & Krizman (77) even philosophized on the significance of these observations in relation to manifestations of early forms of life. Engelhardt (21) believes that some of the conclusions derived from the use of the tracer techniques are somewhat exaggerated.

Orekhovich *et al.* (78) injected tyrosine-carboxyl-C^{14}, glycine-1-C^{14}, and methionine-S^{35}, under sterile conditions, into the protein membrane of eggs. The latter were incubated, and proteins were isolated from egg white, the yolk, and the embryo. Only the proteins of the embryo contained radioactivity. When radio tyrosine was injected into a hen, 12 to 18 hr. before the egg was layed, the egg white had appreciable radioactivity. If the egg was layed 2 days after the injection of radiotyrosine, the yolk, egg white, and the embryo contained radioactivity. The conclusion was made that the incorporation of amino acids into proteins of the egg takes place only during synthesis of the respective proteins. During incubation of the fertilized egg and development of the embryo renewal of the proteins of the egg white and yolk does not take place. Dynamic exchange of amino acids of the proteins of the egg white, the yolk, and the embryonic disk in hen's egg does not exist. Using nonfertilized hen's egg, tyrosine-carboxyl-C^{14}, and methionine-S^{35} Orekhovich *et al.* (79) found that these amino acids are slowly incorporated into the embryonic disk, but not into the egg proteins. Glycine-1-C^{14} was incorporated into the proteins of egg white and yolk, but this may have been a result of decarboxylation of glycine and incorporation of the CO_2-C^{14} into the proteins. However, glycine-2-C^{14} also was incorporated into the proteins and the possibility of decarboxylation of glycine and CO_2 fixation into the proteins is not considered valid. No explanation is offered at this time for the incorporation of glycine into the proteins of non-fertilized hen's egg.

Khesin (80) studied the synthesis of amylase by cytoplasmic granules which were isolated from pancreatic cells. In these cells of the pigeon not less than one half of the amylase is located in cytoplasmic granules. Following the rupture of the granules, amylase can be extracted with butanol. In the pres-

ence of ATP, ketoglutarate, and amino acids the cytoplasmic granules of pancreatic cells synthesize amylase. The synthesis occurs only during incubation of intact granules in a medium which contains all the amino acids which are found in amylase protein, and the synthesis continues for 20 min. During this time the amylase content increases by 3 to 4 per cent. Taking into account proteolysis which occurs during this period, the actual amount of amylase synthesized is about 6 per cent. The synthesis of amylase takes place in the secretory granules during their growth. For this synthesis certain unknown nonprotein compounds are required, and these are synthesized by mitochondria in the presence of ATP and oxygen. The synthesis does not occur under anaerobic conditions. On addition of these unknown products, formed by mitochondria, to the secretory granules the latter can synthesize amylase in the presence of amino acids and in the absence of ATP and oxygen. This indicated to the author that ATP does not participate directly in peptide bond synthesis of amylase, and that this synthesis is not coupled with respiration. The respiration of pancreas cells is accomplished by mitochondria, but the synthesis of amylase protein is induced by secretory granules. The data also indicate that structural inviolability of the cell is not a prerequisite for protein synthesis, and the latter can be accomplished for some time during incubation of separate elements of the cell structure. Khesin (81) studied the synthesis of protein in various structural elements of cells of rat liver, employing methionine-S^{35} and tyrosine-C^{14}. He found that the extent of incorporation of radio amino acids into microsomes was about the same as in the "upper layer" of large granules, and higher than in mitochondria. The possibility that the "upper layer" contains microsomes was not excluded, and the synthesis of protein in the microsomes is considered a distinct possibility. Khesin & Petrashkaite (82) continued the study of protein synthesis in cytoplasmic granules of liver cells. The liver was homogenized and centrifuged at 1,000 r.p.m. The supernatant was centrifuged for 20 min. at 3,000 r.p.m., and several layers were obtained. The upper layer consisted mainly of "light granules" and mitochondria. These were resuspended and recentrifuged. The "light granules" were able to synthesize protein only in the presence of a complete mixture of amino acids and only after certain unknown substances were allowed to be elaborated by the mitochondria. The "light granules" from regenerating liver were more active in this respect than those from normal liver. The extent of protein synthesis by "light granules" was greater than by microsomes which were isolated from the same tissue, and the rate of protein synthesis by a mixture of "light granules" and microsomes proceeded at about the same rate as by "light granules" alone. The authors conclude that liver cells contain specific cytoplasmic granules which are different from microsomes and that these granules are analogous to maturing zymogenic granules of the exocrine cells of the pancreas. Their primary function appears to be synthesis of protein.

Kurokhtina (83) administered methionine-S^{35} to dogs and 22 hr. later

blood was taken from the femoral vein. The specific activity of proteins was determined on total proteins, fibrinogen, euglobulin, pseudoglobulin, and albumin. In another experiment, the activity was determined on globulin and albumin at various intervals, ranging from 0 time to 96 hr., every 1.5 to 3 hours. It was found that the S^{35} of methionine entered globulin faster than into albumin, indicating faster synthesis or renewal of globulin than of albumin. The specific activities of pseudoglobulin, fibrin, and albumin were not the same, being lowest for the albumin. These observations are in accord with those of Miller *et al.* (84) who used lysine-C^{14}, and at variance with Schoenheimer *et al.* (85), who used N^{15}-labeled amino acids, and with Tarver & Schmidt (86) who also used methionine-S^{35}.

Gavrilova & Konikova (87) prelabeled the serum of rabbits and rats with methionine-S^{35}; the serum was dialyzed and serum proteins were isolated. Proteins were also isolated from homogenates of liver, kidney, nuclei of liver, and fibrin was isolated from the blood. The authors report that the proteins of the homologous or heterologous serum of rats and rabbits, labeled with S^{35}, are converted to fibrinogen of the plasma. Intraperitoneal injections produced the same results. Nonisotopic methionine, administered along with serum proteins, which were prelabeled with methionine-S^{35}, did not "dilute" the activity of the isolated proteins.

Volovnik (88) finds that in rats deficient in pyridoxin there is a sharp (45 to 70 per cent) decrease in the incorporation of S^{35} of L, D, or DL-methionine into tissue cystine. The D isomer of methionine yielded less of its S^{35} than the L isomer, which suggests a decrease in the inversion of D-methionine to the L antipode *in vivo*. These data support the suggested participation of pyridoxal phosphate in transamination and transulfuration mechanisms (89).

Cherkes *et al.* (90) studied the relationship between the metabolism of nicotinic acid and the sulfur-containing amino acids. The inclusion of nicotinic acid into the diet of young rats prevented to a significant extent the development of pathological changes in the liver which were induced by an excess of cystine in the diet. The addition of an excess of sulfur amino acids into the diet diminished the amount of methylnicotinamide excreted in the urine. This is not connected with, or induced by, a lowered ability of the rat to synthesize nicotinic acid from tryptophan, nor is it a result of development of a deficiency in labile methyl groups. The system which governs the conversion of cysteinesulfenic acid to cysteic acid (91), which depends on coenzyme III (containing nicotinamide), may be related to this interdependence of cystine and nicotinamide metabolism in the rat.

Shmerling (92) found that the serum albumin of horses cannot be detected electrophoretically in rat embryo blood after injection of horse serum into a pregnant rat. After introduction of rat serum, labeled with methionine-S^{35}, into the blood of a pregnant rat, the embryo blood serum was free of the label. It was concluded that the serum proteins (albumin) of embryonic rats

are formed by the embryo, as well as in the adult rats, by the liver. The placenta does not participate in the synthesis of serum albumin of the embryos.

Khesin (93) found that after the subcutaneous administration of methionine-S^{35} and tyrosine-C^{14} to rats, the labeled amino acids were incorporated at the greatest rate into serum albumin, which was firmly bound in large cytoplasmic granules. The incorporation of amino acids into albumin of submicroscopic granules and centrifugates occurs considerably more slowly. Comparison of the radioactivity curve of a fraction which contained free labeled amino acids, with changes in the radioactivity of albumin of various structural elements of the liver cell showed a direct relationship between the radioactivity of free amino acids and the radioactivity of albumin found in the large granules. The radioactivity of microsomes and of other albumin fractions was not directly related to the radioactivity of free amino acids. It is suggested that the newly synthesized albumin of large granules passes from large granules to other cell fractions, including microsomes. This transfer is not a result of degradation of albumin in large granules, but constitutes a physiological process which occurs during aerobic respiration. Serum albumin of large granules, isolated from rat liver, is not located in mitochondria, but in large granules of a different type, which are analogous to secretory granules of pancreatic cells.

Enzymatic studies.—Titova & Shapot (94) observed that the addition of an enzymatic system, 6-phosphofructokinase+fructose-6-phosphate (which diverts ATP towards phosphorylation of sugar), to isolated cytoplasmic granules of kidney cortex (capable of respiration, phosphorlyation, and renewal of protoplasm), caused a sharp rise in respiration and coupled phosphorylation but a decrease in the extent of incorporation of methionine-S^{35} into proteins and a decrease in the extent of incorporation of P^{32} into phospholipids of the granules. The rate of exchange of phosphorus in RNA was increased. The alteration in protein metabolism in granules as a result of diversion of ATP to another process is regarded as a biochemical model of depletion of a physiological system.

Bresler & Rubina (95) studied the enzymatic transfer of phosphate groups from RNA to fructosemonophosphate. The authors phosphorylated calf pancreas RNA in the presence of ATP and myokinase. Unpolymerized RNA was prepared by the method of Dounce & Kay (96) and myokinase (containing 6-phosphofructokinase) was prepared according to Neifakh *et al.* (97). Phosphorylated RNA was prepared as follows: 10 ml. of the Na salt of RNA (20 mg. dry substance), 10 ml. Na salt of ATP, 2 ml. of 0.03 *M* $MgSO_4$ and 2 ml. of myokinase (about 10 mg. protein) were mixed. The mixture was incubated for 1 hr. at 37°. After cooling, solid NaCl was added to the mixture to a concentration of 1 *M*, and double the volume of 95% ethanol was added. The precipitate contained the Na salt of RNA and some protein of the enzymes. The precipitate was dried, then dissolved in 0.9 per cent NaCl. The

denatured protein was not dissolved, and it was removed by filtration. Na-RNA was again dissolved in 0.9 per cent NaCl and precipitated with ethanol, washed with ethanol, and dried. The material was free of ATP and ADP, and it contained 35 to 65 per cent excess phosphorus. The reaction

$$\text{RNA—P} + \text{Fructose-6-phosphate} \xrightarrow{\text{6-phosphofructokinase}} \text{RNA} + \text{fructosediphosphate}$$

was examined by incubating RNA-P (phosphorylated RNA) [0.2 ml. (2 mg. dry substance)], 0.15 ml. of fructose-6-phosphate (1.5 mg. dry substance), 0.2 ml. of 0.03 *M* $MgSO_4$, 0.1 ml. of 0.6 *M* NaF, 0.4 ml. borate buffer (pH 7.2), and 0.5 ml. of 6-phosphofructokinase containing myokinase. The total volume was 1.55 ml. The mixture was incubated 15 min. at 37°. To the trichloroacetic acid filtrate, neutralized to pH 7.2, 0.1 ml. of versene (final concentration 10^{-3} *M*), 1 ml. of borate buffer (pH 7.2), 1 ml. of 0.25 *M* KCN, and 1 ml. of aldolase (1.5 mg. protein) were added. The mixture was incubated for 10 min. at 37°. The trichloroacetic acid filtrate was hydrolyzed with KOH (equal volume of 1 *N* KOH) at room temperature. Phosphorus was determined in the hydrolyzate. The amount of free phosphate was determined separately in all individual preparations. The results showed that excess phosphorus found in the fructose diphosphate was formed at the expense of phosphorylated RNA, and that the latter, like ATP, possesses high energy phosphate. Seven-minute acid hydrolysis of phosphorylated RNA gave 40 per cent phosphorus in excess of that present in unphosphorylated RNA, and 80 per cent of this excess phosphate was utilized in the enzymatic transfer to fructosediphosphate. Evidence for the presence of a pyrophosphate group at the 5-carbon atom of ribose of RNA is derived from the fact of participation of myokinase and 6-phosphofructokinase in the specific utilization of the phosphate groups in the substrate. For this reason the phosphorylated polymerized RNA is considered an analogue of ATP or ADP. What the specific function of this energy-rich RNA is, remains to be elucidated.

Tatarskaya *et al.* (98) found that the cofactor of thiaminase is highly stable to prolonged hydrolysis with acids and alkali. Various simple, nitrogen-containing compounds, present in tissue extracts, can apparently act as cofactors of thiaminase. Either thiaminases are different in various species, or thiaminase is an enzyme of a new, not yet known type, chiefly because of lack of specificity towards the cofactors. Taurine, as the cofactor of thiaminase of clams (Venus mercenaria?), claimed by Barnhurst & Hennessy (99), is disputed by the authors on the ground that clams contain almost 5 per cent taurine in tissues.

Mel'nikova & Neifakh (100) studied the nature and properties of 6-phosphofructokinase of muscle, which catalyzes the reaction fructose-6-phosphate+ATP→fructose-1,6-diphosphate+ADP. The kinase was obtained as an electrophoretically homogeneous protein [Neifakh *et al.* 97)]

from skeletal muscle of rabbits. Data obtained on sedimentation and by diffusion indicated a molecular weight of 120,000. The enzyme constitutes 0.3 to 0.5 per cent of the wet weight of tissue. The activity of the enzyme depends on free thiol groups and on Mg^{++}. It is inhibited by citrate, adenylic acid, thiol poisons, and 2,6-dichlorophenolindophenol. The kinetics of the enzyme and catalytic constants were determined: $K_M = 1 \times 10^{-3}$ M (using fructose-6-phosphate); turnover number was 350; $Q_T = 3900$; Q for fructose-phosphokinase was 35 to 38 per mg. dry tissue. The energy of activation of the fructose phosphokinase reaction was 5200 cal./mole. The activity of fructosephosphokinase per gram of tissue in rhabdomyokinase is 1.6 times higher than in skeletal muscle of rats. The authors suggest that phosphofructokinase as the "slowest" enzyme in the glycolytic spectrum of enzymes limits the rate of glycolysis in the muscle.

Gershenovich & Krichevskaya (101) found that L(+)-glutamic acid, and, better still, DL-glutamic acid, protects rats and mice against increased oxygen pressure. Convulsions set in at $Q_{O_2}{}^{O_2} = 1.14$. Glutamic acid, especially the racemate, supported high levels of tissue (brain) oxidation, which was depressed, in the absence of glutamate, by high oxygen tension. Neither aspartic acid nor alanine had any effect on the oxidation of brain tissue or on protection of animals against high oxygen tension.

Rykhlik *et al.* (102) studied, *in vitro*, the synthesis of proteases and amylase of the pancreas of mice. The synthesis required O_2, and was inhibited by 2,4-dinitrophenol. Maximum synthesis occurred in the presence of casein hydrolyzate fortified with tryptophan. Azaserine (O-diazoacetylserine) strongly inhibited protein synthesis. Methioninesulfoximine also was inhibitory. Ethionine, however, did not inhibit protein synthesis even in a concentration of 500 μg. per ml. This finding is contrary to that of Simpson *et al.* (103) but in accord with that of Farber *et al.* (104), who found that ethionine *in vivo* had no effect on the activity of pancreatic enzymes five to six hours after the administration of ethionine to animals. Chloramphenicol, in a concentration of 500 μg. per ml., inhibited protein synthesis.

Ivanova (105), by a kinetic study of catalase decomposition of H_2O_2, showed that the rate of hydrogen peroxide decomposition is increased with an increase in concentration of catalase. The rate of reaction is also increased with an increase in H_2O_2 concentration up to a certain limit, then decreases. Evidence is available to show that this decrease in the reaction is probably due to inactivation of catalase which takes place during decomposition of H_2O_2. The data do not agree with equations worked out by Kovosev (106) or with that of Afanasiev (107), but are in complete accord with the equations of Michaelis-Menten. Kovosev (106) and Afanasiev (107) incorrectly calculate the initial rate of the reaction.

Kritsky & Melik-Sarkisyan (108), by fractional precipitation of salt solutions of rat liver homogenates, isolated an electrophoretically homogeneous protein which possessed transpentosidase and nucleosidophosphorylase

activities towards purine ribosides. In the same liver, another protein (named transpentosidase B in contrast to transpentosidase A of another fraction) was isolated which possessed enzymatic specificity.

Trufanov & Popova (109) showed that coenzyme A (Co-A) can be readily synthesized by brain homogenates in the presence of pantothenic acid, ATP, cysteine, and Mg^{++}. Liver, kidney, heart, and muscle preparations were ineffective in Co-A synthesis under similar conditions. Addition of both thiamine and pyruvate to brain homogenates increased the extent of Co-A formation. Thiamine and pyruvate, added singly, were ineffective. Brain homogenates of rats, deficient in pantothenic acid, synthesized more Co-A on addition of pantothenate than normal rat brain homogenates did under similar conditions. Benign brain tumors synthesized more Co-A than did malignant brain tumors. Irradiation of guinea pigs with x-rays for 10 min. (500 r) induced a decrease in Co-A synthesis in brain homogenate.

Sinitsina (110) finds that the extent of oxidative deamination of L-glutamic acid in liver homogenates and the extent of animation of ketoglutarate by liver and kidney slices are not decreased by deficiency in pyridoxin in rats. In liver and kidney slices of pyridoxin-deficient rats, incubated with pyruvate and ammonia, the amount of amino acids present was sharply reduced. Glutamic acid was formed in increased amounts, but alanine was either absent or present in traces. The synthesis of phenylalanine and histidine from phenylpyruvate and imidazole lactic acid, respectively, by liver and kidney slices ceased and it was not increased by the addition of ammonia. In tissues of animals in which avitaminosis-B_6 decreases the activity of aminopherases there is a decrease in oxidative deamination (glutamic acid excepted) and a decrease in amination of α-keto acids (ketoglutarate excepted). Transamination appears to be the exclusive process in this reaction.

Sorvachev (111) observed that β-alanine, added to homogenates of kidney cortex, liberated ammonia and CO_2 in phosphate buffer in the presence of O_2. In bicarbonate buffer the extent of formation of ammonia and CO_2 from β-alanine was about one-twentieth of that found in phosphate buffer. Ammonia was rapidly formed from phospho-β-alanine in the presence or absence of O_2. Ethylamine and hydroxyethylamine were not the intermediate products in β-alanine degradation, and they were not deaminated by kidney homogenates. The nature of the deaminated product of β-alanine is as yet unknown.

Beresovskaya (112) reports that lecithinase C and alpha toxin of *B. perfringens* are identical. Normal guinea pigs contain lecithinase C in the brain in bound form. On administration of alpha toxin to guinea pigs, lecithinase-C activity in a free form was found in the kidney, muscle, brain, and blood. In brain homogenates all of the activity of lecithinase C was in the precipitate, and none was found in the supernatant after centrifugation. On addition of alpha toxin to homogenates, the activity was found in the supernatant only. The conclusion is made that the administration of apha

toxin *in vivo* or the addition of it to homogenates *in vitro* leads to transformation of bound lecithinase C to a free form. Druzhinina *et al.* (113) showed that lecithinase C, present in normal animals is identical with lecithinase in alpha toxin. The absence of lecithinase C from the lung is surprising because, the lung is one of the organs which is rapidly and extensively affected by alpha toxin.

Garkavi (114), in accord with the conclusions of Yudaev (115) and Parshin & Gorukhina (116), found that liver slices and homogenates of muscle of rats do not methylate carnosine in the presence of methionine. Carnitine, in lieu of methionine, also failed to effect the methylation of carnosine or glycocyamine. Carnitine did not affect the respiration of pigeon muscle nor did it affect the binding of inorganic phosphate by the tissues. The content of carnitine in muscle tissue of cows, rats, chickens, pigeons, ravens, frogs, and fish covered the range of 48 to 260 mg. per cent for the different species.

Leutskiĭ & Naumchuk (117) observed that the livers of rats, kept for 70 days on diets which were free of NaCl, showed a reduced capacity to methylate nicotinamide (9.8 μg. per gm. tissue in deficient livers, and 37.4 μg. per gm. tissue in normal controls). The authors suggest that deficiency in dietary NaCl creates an unfavorable situation for the metabolism of certain water-soluble vitamins, citing the observation of Handler & Dann (118) that the life span of nicotinic acid-deficient dogs could be prolonged by administration of 0.9 per cent salt solution.

Yudaev (115) produced evidence that anserine is synthesized *in vivo* from β-alanine and methylhistidine. The appearance of the deuteriomethyl group of methionine in anserine of rabbit muscle is not a proof that anserine is synthesized by methylation of carnosine, because the deuteriomethyl group of anserine could have arisen from previously methylated histidine.

Gulevich (119) suggested that carnosine is synthesized in animals via one of two paths: decarboxylation of aspartylhistidine, or condensation of β-alanine with histidine. Parshin & Gorukhina (116) now report evidence that carnosine is synthesized from β-alanine and histidine. Dogs with Eck fistulae were used, and the content of muscle carnosine and anserine was determined 50 days after the fistulae were established. The values were compared to those found in muscle of normal dogs. Enzymatic activities of the livers of experimental and control dogs for histidine deaminase and urocanase were also measured. Carnosine and anserine were determined enzymatically by the method of Parshin (120). An Eck fistula in dogs reduced sharply the content of carnosine and anserine in the muscle without affecting the activities of histidine deaminase and urocanase. A deficiency in histidine and methylhistidine, due to unavailability of these compounds from the digestive tract to the liver, is the responsible cause. Koldaev (121) and Hunter (122) have previously shown that starvation or removal of protein from the diet sharply reduces muscle carnosine content. Parshin & Gorukhina (116) conclude from their experiments that carnosine and anserine are synthesized

in the liver. On the basis of previous data on the enzymatic cleavage of methylhistidine (123), and on the basis of results obtained on subcutaneous administration of histidine and of products of acid hydrolysis of anserine (115), the authors (116) conclude that carnosine is formed in the liver via condensation of β-alanine and histidine, and that anserine is formed in the liver from β-alanine and methylhistidine. The latter is formed in the liver by methylation of histidine. A method for chromatographic localization of carnosine and anserine of muscle tissue and of estimation of their content was developed by Yudaev (124). Goryachenkova (125) established that the enzyme from garlic, allinase, which forms allicin, is a pyridoxal phosphate protein, which catalyzes the conversion of alline to allylsulfenic acid and α-aminoacrylic acid. The conversion of allylsulfenic acid to allicin and of aminoacrylic acid to pyruvic acid and ammonia take place spontaneously.

Goryachenkova (126) demonstrated that joint function of pyridoxal phosphate and flavin adenine dinucleotide is required for the action of diamine oxidase (histaminase). On the basis of earlier suggestions (89, 127), she proposes that the oxidation of diamines takes place via amino group transfer from substrate to protein-bound pyridoxal phosphate (transamination), followed by dehydrogenation and deamination of the resulting pyridoxamine phosphate by the flavoprotein moiety of the enzyme.[2] The experiments, however, do not rule out the possibility of an alternate mechanism of action of diamine oxidase in which transamination step is bypassed. This latter mechanism is that suggested by Werle & Pechman (127), and the one which includes a transamination step is that of Braunshtein & Shemyakin (89), previously reviewed by Fried & Lardy (128).

Gorkin & Kondrashova (129) reported that "Necrosin" of Menken (130) is identical with macrocytase, a proteolytic enzyme which was discovered and described by I. Mechnikov in 1901 (131) and detected by Tarasevich (132) in pus exudates at relatively late stages of necrosis. "Necrosin" of necrotic exudates has been shown to be a proteolytic enzyme of trypsin type with an optimal pH of 7.7 to 8.2. It splits several proteins and possesses fibrinolytic activity. The authors conclude that the ability of "necrosin" to induce necrosis is a function of its proteolytic activity.

Efimochkina (133) reported that in surviving liver slices the activity of the tryptophan-peroxidase system (TPS), on addition of L-tryptophan, is rapidly increased. Under similar conditions, the activity of TPS in liver extracts does not increase, and the oxidation of tryptophan proceeds at a constant rate. In the absence of tryptophan the activity of TPS in slices or extracts rapidly falls. In slices, the TPS activity after a fall can be restored

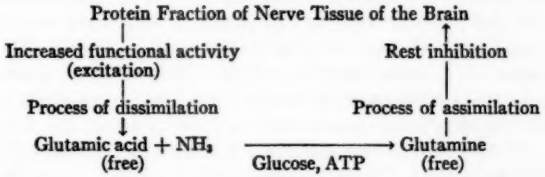
[2] A. N. Davison (*Biochem. J.*, 64, 546, 1956) recently reported, in confirmation of these data, that pig-kidney diamine oxidase and histaminase are a single enzyme with pyridoxal phosphate as the coenzyme. The participation of flavin-adenine dinucleotide at some stage in the enzyme reaction is not excluded.

by the addition of L-tryptophan; in extracts, the loss of TPS activity is irreversible. To the author's knowledge, this is the first demonstration in isolated mammalian tissue of adaptive enzymatic changes which are probably similar to those observed in bacteria or in whole animals (arginase, glutaminase, glucuronidase) by other workers. This adaptationof TPS to tryptophan is inhibited by dinitrophenol and other compounds which do not inhibit respiration, but which dissociate its dependence on generation of high-energy phosphate for the biosynthesis of enzyme protein.

Vilenkina (134) reported that an enzyme, serinase, from liver extracts of guinea pigs reacts only with L-serine, giving glycine and an aldehyde (formaldehyde). Folinic acid is the cofactor of serinase. Crude preparations of serinase were activated by homocysteine, but this effect of homocysteine was thought to be nonspecific. While this work was in press, Blakly (135) reported experiments with purified preparations from pigeon liver which indicated that pyridoxal phosphate and tetrahydrofolic acid are the cofactors in the transformation of serine to glycine.

Biochemistry of the brain.—In 1955 Palladin (136) extensively reviewed the available experimental data on the biochemistry of the brain. In this review he described pioneer and recent work performed at the Ukrainian Institute of Biochemistry of the Academy of Science, and by other workers in the Soviet Union and outside. He stressed the importance of preserving conditions which prevail *in vivo* for the elucidation of the functional biochemistry of the brain. In the opinion of Palladin (136), experiments *in vitro* can yield information concerning only the mechanisms of enzymatic reactions involved and not their functional significance in the intact animal. Work, *in vitro*, with nerve tissue, in spite of the great and immediate sensitivity of the tissue to manipulation of almost any kind, is nevertheless capable of producing valuable information which occasionally is qualitatively similar to that obtained *in vivo*, as Palladin clearly shows. This review of Palladin (136) is available in Russian and French. It has been reported and confirmed that ammonia is formed by nerve fibers during excitation (137), and that the source of this ammonia is not adenylic acid or its derivatives or glutamic acid amino nitrogen (138). Vrba (139) presents experimental data to show that the source of ammonia arising during excitation of rat brain is glutamine in the proteins of nerve tissue, i.e., bound glutamine. The free glutamine found in the brain is only a reflection of exchange processes in the brain and it does not represent an active center of functional activity of the brain tissue. The center of nerve activity is in the structural, nonextractable component parts of the nerve tissue. Ammonia formation by nerve tissue is intimately connected with its functional activity, and, therefore, is a result of the participation of nerve tissue proteins in the functional activity. Experimental data show that during nerve excitation deamidation of brain proteins takes place along with further degradation of the proteins themselves. During inhibition of nerve activity reamidation occurs along

with resynthesis of brain proteins. The connecting link between the processes of excitation and inhibition (rest) is the system glutamic acid→glutamine which is situated at a strategic biochemical locus of the continuous cycle of degradation and resynthesis of brain proteins. This is based on the observation that during the excitation process there is a parallel decrease in the concentration of free glutamic acid and an increase in glutamine. During inhibition, under physiological conditions, free glutamine concentration is decreased and protein nitrogen of the brain increased. The following scheme illustrates this proposed "Hypothesis of the glutamic acid cycle":



The basis of excitation and inhibition are undoubtedly complex chemical processes, and the proposed theory is offered only as a contribution to a partial understanding of these mechanisms. It is also pointed out that the well-known acetylcholine neurochemical theory of excitation, valuable as it is, does not take into account the formation of ammonia during the active phase of nerve (140). A similar omission is evident in the so-called ionic theory of transmission of nerve impulses (141).

Cancer.—Davidova (142) reported that the growth of transplanted tumors (sarcoma C-45 and sarcoma M-1) in rats leads to an increased content of DNA and RNA in the liver, kidneys, lungs, and spleen. In the tumor, with growth, no increase in DNA and RNA was noted. Growth of transplanted tumors leads to changes in the relative content of nitrogenous bases in nucleic acids, and these changes are not parallel or identical in tumors and tissues of the host. The percentage content of thymine in DNA in all tissues of the host decreases with tumor growth, while in the tumor itself thymine increases. The content of adenine in nucleic acids of the host tissues is greater than in normal rats; the content of guanine is lower. In tumor, the adenine content of nucleic acids is almost the same; that of guanine increases. These data, on the whole, confirm the observations of Lombardo *et al.* (143), except that the latter workers found that guanine and adenine of nucleic acids from tissues of tumor-bearing animals increased, the increase in guanine being greater than that of adenine.

Kusin & Davidova (144) found that in rabbits bearing Brown-Pierce tumors, there is a change not only in nucleic acid content but in the relative content of the nitrogenous bases in the nucleic acids. Particularly striking was the increase in thymine content of DNA in tissues of tumor-bearing rabbits, particularly in liver and tumor. DNA of spleen in tumor-bearing rabbits

contained more adenine. The combined guanine and xanthine content in DNA of tumor-bearing rabbits was greater in all organs, except in testicle DNA, where it was lower than in controls.

Kusin *et al.* (145) prepared from animal and human tumors and from the blood of tumor-bearing animals a water-soluble, dialyzable, material of unknown composition which markedly inhibited the activity of liver catalase and Co-A of normal rats. The preparation, injected intraperitoneally into normal animals, lowered the activity of liver catalase and Co-A. Previously, Nakahara & Fukuoka (146) prepared similar material from extracts of tumors which lowered the activity of liver catalase of normal animals. Kusin & Tchudinova (147) further studied this material. The toxin was adsorbed on anion exchanger MG-1, and eluted with 4 per cent ammonia. Chromatography on paper, with butanol-ethanol-water (50:15:35) as solvent, gave a spot with an R_f of 0.27 which was detected under ultraviolet light. Eluted, it was active in depressing the activity of liver catalase. Electrophoresis on paper at pH 6.98 moved the substance towards the cathode. The concentrates were precipitated with chloroplatinate, and a crystalline chloroplatinate, with a M.P. of 218°–225°, was obtained, It was decomposed with H_2S in HCl solution, and the filtrate, containing the active material, showed an absorption maximum at 260 mμ. The compound reacted with diazobenzenesulfonic acid (red) and with hydroxylamine hydrochloride (red). Free amino groups were absent. On analysis, the empirical formula for the chloroplatinate was found to be $C_6H_{19}N_5O_6 \cdot Pt_2Cl_8$. The compound contains either a purine or azoiminopyrimidine ring.

Belousova (148) studied the synthesis of nucleic acids by homogenates and by the isolated nuclei of normal and malignant cells. She finds that for the synthesis of nucleic acids by these systems the necessary substrates are ATP, malic acid, and RNA or products of its degradation. Common substrates for the synthesis of DNA and RNA of the cell nucleus are acid-soluble ribonucleotides and ATP. The synthesis of DNA is connected with growth and cell division and for this reason its synthesis proceeds intensively, not only in nuclei of malignant cells, but also in nuclei of normal, rapidly proliferating, tissue, such as the intestinal mucosa. The nuclei of liver cells did not synthesize DNA. A significant characteristic of the nuclei of malignant cells was found to be their ability to synthesize DNA at low substrate concentrations, the latter being too low for the synthesis of DNA by normal nuclei. Along with the synthesis of DNA in malignant nuclei, under definite conditions, there was observed an intensive synthesis of RNA, which probably took place in nucleoli. DNA synthesis, however, was the dominant process, and at low substrate concentration it favorably competed with RNA synthesis.

Bergol'tz (149) proposes a neuroenzymatic hypothesis of malignant growth; the paper contains a thorough review of pertinent experimental data.

Kleova & Roskin (150) thoroughly reviewed the available information

on anticancer antibiotics; analysis of the evidence indicates that cancer cells are labile under the action of antiblastomic agents which are derived from certain bacteria. Nicolau (151) discussed the available information and the newer data by the author in regard to the virus theory of etiology of benign and malignant tumors, and arrived at a conclusion which favors the virus theory as the *prima causa* of malignancy in animal and human neoplasms.

Ryzhkov (152) proposed that virus particles can be considered as virospores on the basis of the following considerations: (*a*) structural properties of virus particles, namely, the existence of protective protein membranes; (*b*) stability of virus particles; (*c*) position of virus particles in the cycle of virus development; (*d*) absence of respiration and biochemical activity of virus particles *in vitro;* (*e*) preservation of activity and absence of alterations in the progeny of virus particles after benzoylation, acetylation, etc.; (*f*) apparent absence of nucleic acids from viruses as shown by staining with dyes; (*g*) apparent inertness of virus particles, not only *in vitro* but also inside the cells after the developmental cycle of the virus is completed.

Reviews and discussions.—Makarov (153) critically examined the available literature on the theory of the constancy of composition of cellular DNA, and expressed doubt regarding its validity. Bychkov (154) reviewed the subject of mucopolysaccharides and mucoproteins of the eye, and concluded that mucoproteins and mucopolysaccharides as group substances in general, and as components of the eye in particular, are of great physiological importance.

Budnitskaya (155) classified all carotenoids into five basic groups: carotenoids proper (hydrocarbons); hydroxyl-containing carotenoids; carbonyl-containing carotenoids; carotenoids which are derived from carbonic acids; and imperfectly characterized carotenoids. Grischenko (156) reviewed various theories of muscular contraction, and concluded with a proposition that the work performed by muscle during a single contraction is derived from the swelling of actomyosin and from the dehydrated ATP, which in the process of dehydration acquires free energy. El'piner (157) discussed the physicochemical phenomena on the surfaces of cells, tissues, and organisms. The reviewed material indicated that on the surface layer, dividing different phases, there is more then an alteration of the orientation of molecules of various biologically active compounds. Depending on various contions, there exists an interaction of a definite nature between the molecules of the liquid phases as well as between the molecules which constitute the monolayer. The composition of the monolayer, as well as its biological activity, depends on the nature of these interactions. In the functioning organism the direction of the exchange processes (regulated by the central nervous system in higher organisms) is determined, in part, by the alteration of the composition, and, perhaps, the nature of the enzymatic activity or other biological activity of the surface layers of the living membranes. In, or on, the surface layers occurs the concentration and the interaction of various compounds, their degradation, and their synthesis.

Beresov (158) reviewed the modern views on the processes of urea formation in animals, indicating that the immediate source of one of the nitrogen atoms of urea is ammonia which enters the cycle of urea formation during synthesis of citrulline from ornithine. The source of the second nitrogen atom of urea is the amino group of aspartic acid, at the expense of which citrulline is converted to arginine. These data are in accord with the general concept of Braunshtein and co-workers which proposes that the nitrogen of all amino acids and of other nitrogen-containing compounds convertible to urea *in vivo* must, prior to its availability for urea formation, be either free ammonia (directly or via transamination) or be converted via various pathways to the amino nitrogen of aspartic acid.

Lopushanskiĭ & Lopushanskiĭ (159) discussed the energetics of biological syntheses and concluded that "no one ever experimentally proved the theoretically unprovable endergonicity of biological syntheses which contradicts the laws of thermodynamics and experimental data." According to the authors (159), such concepts are antiscientific, idealistic, etc. On the other hand, the concept of Tauson (160) about exergonicity of biological syntheses is experimentally sound, and, besides, it rests on objective laws of thermodynamics.

Kotel'nikova (161) earlier indicated the unconvincing nature of Tauson's (160) postulates, as well as the theory of exergonicity of biological syntheses, on the ground of contradiction with modern developments in biochemistry. In another article, Kotel'nikova (162) points out that the theory of Tauson (160) is only a theory and not a proven fact, and if the Lopushanskiĭs only looked more deeply into the meaning of the laws of thermodynamics it would be found that the views of those who subscribe to the endergonic nature of biological syntheses do not necessarily contradict the laws of thermodynamics. Braunshtein (163) suggests that certain general concepts, discussed by Lopushanskiĭ & Lopushanskiĭ (159), are correct, but rather self-evident and do not require extensive discussion. These are the applicability of the second law of thermodynamics to living organisms, and to any biochemical process of the isolated system. One can agree with the Lopushanskiĭs that these considerations are not always expressed in biological literature (including Soviet authors), leading to inaccurate and uncertain generalizations which, in turn, may produce errors and methodologically erroneous conclusions. According to Braunshtein (163), the other concepts of the Lopushanskiĭs which lead them to erroneous views, stem mainly from a poor understanding of biochemical concepts and experimental findings.

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CHEMISTRY OF THE CARBOHYDRATES[1,2]

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Following the pattern of recent years, this review deals only with a few topics selected from the rapidly expanding literature of carbohydrate chemistry.

Isolations and Syntheses

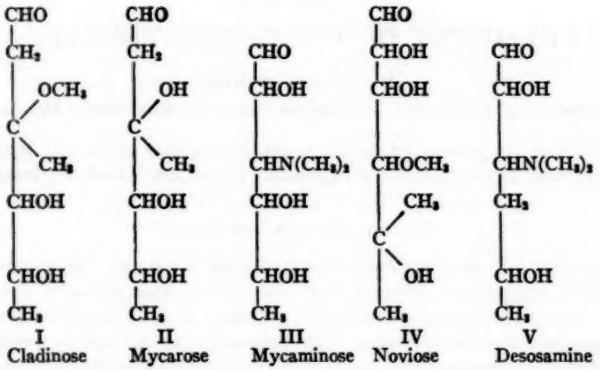
Isolations.—It has long been assumed that the sugar component of the pyrimidine nucleosides derived from deoxyribonucleic acids is 2-deoxy-D-ribose. This assumption has now been verified (1, 2, 3) by identification of the sugar released by hydrolysis following reduction of the pyrimidine nucleus of the nucleosides. The sugar moiety of the nucleosides (spongothymidine and spongouridine) found in certain sponges (4, 5) has been identified as D-arabinose (2, 5). Synthetic 3-β-D-arabofuranosyluracil (6) is identical with the natural spongouridine. A 3-ketopentose, presumably *erythro*-3-pentulose, is formed by the action of mouse spleen extract on D-ribose 5-phosphate (7). 3,6-Anhydro-D-galactose has been isolated from a hydrolysate of *k*-carrageenin (8), and is believed to alternate with D-galactose 4-sulfate in the algal polysaccharide structure (9). The first examples of naturally occurring aldoheptoses, L-*glycero*-D-*manno*-heptose (10, 11) and D-*glycero*-D-*gala*-heptose (12), have been identified from hydrolysates of bacterial polysaccharides.

Antibiotic substances elaborated by various species of *Streptomyces* continue to provide unusual sugar and aminosugar structures: cladinose (I) from erythromycin (13); mycarose (II) and mycaminose (III) from carbomycin (Magnamycin) (14, 15); noviose (novobiose) (IV) from novobiocin (16, 17); desosamine (pikrocin) (V) from erythromycin (18), pikromycin (19), narbomycin (20), and methmycin (21); rhodosamine (isomeric with desosamine) from rhodomycin (22); 3-amino-3-deoxy-D-ribose from puromycin (Achromycin) (23); and 2-amino-2-deoxy-D-gulose from streptothricin and streptolin B (24). The L-*lyxo* configuration has been assigned to noviose on the basis of qualitative rules of optical rotation (25).

It is understandable that confusion in trivial names may arise when several research groups independently and almost simultaneously discover the same antibiotic, and are faced with the necessity of coining a trade name for registration. For example, novobiocin was originally named streptonivicin,

[1] The survey of the literature pertaining to this review was completed in October, 1956.

[2] The following abbreviations and symbols are used in this chapter: ADP and ATP for adenosinedi- and triphosphate; CoA for Coenzyme A; DCC for dicyclohexylcarbodiimide; DPN and TPN for di- and triphosphopyridine nucleotide; FAD for flavin adenine dinucleotide; UDP and UTP for uridine di- and triphosphate; UDPG for uridine diphosphate-glucose; and UDPGal for uridine diphosphate-galactose.



cathomycin, and cardelmycin by three independent discovering groups. It is unfortunate that the name novobiocin has led to the introduction of the name novobiose (16), which clearly indicates a disaccharide to the sugar chemist, for the monosaccharide of the antibiotic. The alternative name noviose (17) is preferable.

The occurrence of the (1→3)-linkage in starch has been revealed by the isolation of nigerose (3-O-α-D-glucopyranosyl-D-glucose) after the hydrolysis of amylopectin under conditions of negligible reversion (26). The disaccharide is hydrolyzed by intestinal extracts (27). 5-O-α-D-Glucopyranosyl-D-fructose (leucrose) has been identified as a product of the action of dextransucrase on sucrose (28). 1-O-β-D-Glucopyranosyl-D-mannitol, found previously in algae (29), has been isolated also from laminarin (30). O-α-D-galactopyranosyl-(1→6)-O-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside (planteose), found previously in seeds of various *Plantago* species (31, 32), has been detected also in seeds of tobacco, *Nicotiana tabacum* (33). The trisaccharide (solatriose) of the alkaloid glycoside α-solanin, from *Solanum demissum*, has been identified as 2-O-α-L-rhamnosyl-3-O-β-D-glucopyranosyl-D-galactose (34). Interest in the bifidus factor (35) has stimulated the examination of the oligosaccharide fraction of human milk (36, 37). The presence therein of O-α-L-fucopyranosyl-(1→2)-O-β-D-galactopyranosyl-(1→4)-O-D-glucose (38, 39) and O-β-D-galactopyranosyl-(1→3)-O-2-acetamino-2-deoxy-β-D-glucopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-O-D-glucose (40, 41) has been established. In addition, an isomeric L-fucosyllactose (39) and a tetrasaccharide of gross structure L-fucose-D-galactose-D-glucose-L-fucose (42) have been detected. The bifidus-active disaccharide 4-O-β-D-galactopyranosyl-*N*-acetyl-D-glucosamine has been isolated from meconium (43) and hog mucin (44).

Methylated sugars have been found in nature principally as constituents of the cardiac glycosides and of certain plant polysaccharides. The cardiac glycosides have provided a new, crystalline 6-deoxy-3-O-methylaldohexose,

"acofriose" (45). The sugar appears to be the epimer of L-thevetose (6-deoxy-3-O-methyl-L-glucose) and to be, therefore, 3-O-methyl-L-rhamnose. Partially methylated sugars of as yet undetermined structure have been identified in hydrolysates of soil, peat, and compost (46). They are presumably of microbial origin since they are not found in fresh compost. Apparently the first example of a naturally occurring, methylated aldopentose, 2-O-methyl-D-xylose, has been isolated from a hydrolysate of plum leaf hemicellulose (47). Jute seed has been reported by Khalique & Ahmed (48) to provide a glycoside which, upon hydrolysis with concentrated hydrochloric acid, yields a 2-deoxy-3-O-methylaldopentose, "corchsularose." The latter, upon demethylation, is reported to give 2-deoxy-D-ribose (49). It is incredible to this reviewer that 2-deoxy-D-ribose and its 3-methyl ether could survive the procedures recorded for the isolation and demethylation.

The glycoside α-D-galactopyranosyl-2-glycerol, which had previously been found in the red alga *Irideae laminaroides* (50), has now also been detected in *Gelidium pristoides* and *Gracilaria confervoides* (51). The isomeric β-D-galactopyranosyl-1-glycerol has been isolated from the lipocarbohydrate fraction of wheat flour, where it is accompanied by 6-0-α-D-galactopyranosyl-β-D-galactopyranosyl-1-glycerol (52). The amazingly sweet (300 x sucrose) glycoside stevioside, which was first isolated from the Paraguayan shrub *Stevia Rebaudiana* Bertoni many years ago, has been the subject of recent reinvestigation. The glycoside contains three D-glucose residues, of which one is esterified at C-1 by a highly hindered carboxyl group of the aglycone, and two constitute a sophorose (2-O-β-D-glucopyranosyl-D-glucose) moiety in glycosidic combination with the aglycone (53, 54, 55). The aglycone of stevioside is apparently a diterpenoid acid with a 2,11-cyclopentanoperhydrophenanthrene skeleton (56).

The isolation of L-guluronic acid from alginic acid (57) and the chromatographic identification of iduronic acid from chondroitin sulfate B (58) have been reported.

Syntheses.—This section includes only a selected list from the many important syntheses described in the carbohydrate field during the past few years.

The outstanding synthetic achievement recorded recently is the long-sought chemical synthesis of sucrose. Lemieux & Huber (59) isolated sucrose octaacetate in 5.5 per cent yield by chromatographic separation of the products obtained through reaction of 1,2-anhydro-α-D-glucopyranose triacetate (Brigl's anhydride) with 1,3,4,6-tetra-O-acetyl-D-fructose followed by acetylation. The result, which had been anticipated on the basis of a conformational analysis (60) of the properties of the anhydride, provides synthetic evidence for the α-D-glucosidic linkage in sucrose to bolster similar evidence from enzymatic (61) and optical rotation (62) considerations.

The first known ketononoses, D-*erythro*-L-*manno*-nonulose and D-*erythro*-L-*gluco*-nonulose, have been prepared by Wolfrom & Wood (63) through application of the diazomethane synthesis to the acid chlorides of the corre-

sponding octonic acids. The observed nonfermentability of the two sugars is of interest in view of the reported fermentation of an aldononose prepared by Fischer & Passmore from D-*erythro*-L-*manno*-octose (64). The oxo reaction has been studied as a means of lengthening the carbon chain of the sugars (65, 66). Thus, D-galactal triacetate, when allowed to react under pressure in benzene solution with carbon monoxide and hydrogen in the presence of dicobalt octacarbonyl, gave a mixture of products from which, after deacetylation, a crystalline anhydrodeoxyheptitol was obtained. The coupling of aldoses through piperidine-catalyzed condensation with diethyl acetonedicarboxylate to give long-chained "sugars," $2RCHO + EtO_2C-CH_2COCH_2-CO_2Et \rightarrow RCH=C(CO_2Et)COC(CO_2Et)=CHR$, has been reported (67). The condensation of aldoses with nitromethane to produce higher-carbon sugars, usually catalyzed by sodium methoxide in methanol solution, may also be realized in aqueous alkali (68). The yields by the latter method are appreciably lower.

The MacDonald-Fischer method of sugar degradation via the disulfones has been extended to D-fructose (69, 70), *myo*inosose-2 (70), L-rhamnose (71), D-galactose, and D-glucose (72). Rice & Johnson have reported an interesting degradation of acetylated aldonic acids through treatment of their silver salts with bromine (73), or of their acid chlorides with silver oxide and bromine (74). The resulting decarboxylation is essentially quantitative and the product is the next lower, fully-acetylated *aldehydo*-1-bromoaldose. Glycol-cleaving oxidants have been used extensively for the preparation of lower-carbon sugars and uronic acids from unsubstituted higher sugars and uronic acids, respectively. Oxidation of D-glucose with periodate provides D-glyceraldehyde (75), while lead tetraacetate has been employed for the following degradations: D-mannose or D-glucose →D-arabinose, and D-galactose →D-lyxose (76); D-glucose →D-erythrose, and L-arabinose →L-glyceraldehyde (77); D-fructose →D-glyceraldehyde, and L-sorbose →L-glyceraldehyde (78); D-glucurono-γ-lactone →D-*arabo*-penturonic acid, D-galacturonic acid →D-*threo*-tetruronic acid, and potassium D-glucuronate →D-*erythro*-tetruronic acid (79).

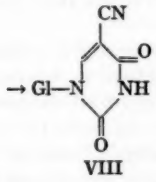
D-*gluco*-Dialdohexose, obtained by the controlled reduction of D-glucurono-γ-lactone with sodium borohydride, or by the Nef reaction on 1,2-O-isopropylidene-6-deoxy-6-nitro-D-glucofuranose, has been added to the list of known dialdoses (80). The pyrolytic rearrangement of sugar alkyl xanthates to the corresponding S-(S-alkyl dithiocarbonate) esters, $=CHO—CS—SR \rightarrow =CHS—CO—SR$, followed by reductive desulfurization of the latter, has been employed in the synthesis of 2-deoxy-D-*erythro*-aldopentose (2-deoxy-D-ribose) (81) and of 3-deoxy-D-*ribo*-aldohexose (3-deoxy-D-glucose) (82). In relation to the latter synthesis, a greatly improved method of preparing 1,2:5,6-di-O-isopropylidene-D-glucofuranose 3-(S-methyl xanthate) is worthy of note (83).

The first example of an isosaccharinic acid of other than six-carbon chain

length, racemic 2,4-dihydroxy-2-hydroxymethylbutyric acid, has been obtained by the action of lime-water on xylobiose (4-O-β-D-xylopyranosyl-D-xylose) (84) and the related trisaccharide, xylotriose (85). The action of alkali on D-fructose has been found to yield, among other products, DL-sorbose, DL-allose, and DL-psicose (86). Similarly, DL-sorbose has been identified among the products from the action of a strong base resin on D-glucose (87). To the several disaccharides recently identified from the acid-catalyzed reversion reaction of D-glucose have been added 3-O-α-D-glycopyranosyl-D-glucose (88) and 5-O-β-D-glycopyranosyl-D-glucose (89). Analogous to the behavior of D-glucose, D-galactose gives 6-O-α-D-galactopyranosyl-D-galactose as a major product upon acid reversion (90). The first example of a 1,7-anhydroaldoheptose, 1,7-anhydro-D-*glycero*-β-D-*gulo*-heptopyranose, has been reported by Stewart & Richtmyer (91) in their continuing studies of the anhydrization of sugars in acid solution.

A new synthesis of pyrimidine nucleosides has been achieved (92) through the condensation of glycosylamines ($Gl.NH_2$) with α-cyano-β-ethoxy-N-carbethoxyacrylamide (VI) to yield the linear compounds (VII), followed by cyclization of the latter to the 5-cyano-1-glycosyluracils (VIII). An interesting synthesis of 3-amino-3-deoxy-D-ribose, the sugar of the antibiotic puromycin, from D-xylose proceeds through all four D-aldopentose configurations

$$\underset{\text{VI}}{EtOCH{=}C(CN){-}CONH{-}CO_2Et} \xrightarrow{Gl.NH_2} \underset{\text{VII}}{Gl.NHCH{=}C(CN){-}CONH{-}CO_2Et}$$



VIII

(93). The N-phthalyl blocking group (94), which completely neutralizes the amino group, and the N-2,4-dinitrophenyl blocking group (95), which is stable to acid but removed by alkali, have been used to advantage in syntheses involving aminosugars.

The last two possible inositols, the 1,2,3/4,5,6 isomer (*neo*-inositol) (96) and the all-*cis* isomer (*cis*-inositol) (97), have been synthesized by Angyal and his associates. With the synthesis of L-*allo*- and D-*talo*-heptulose by the action of *Acetobacter suboxydans* on the appropriate alcohols (98), at least one enantiomorph of all the possible heptuloses is now known. 2,5-Anhydro-L-arabinose, the first known example of its class, has been prepared (99). Glycosides of the anhydropentose are remarkably unstable, being hydrolyzed in distilled water at room temperature. The natural, branched-chain sugars apiose and cordycepose have been synthesized in racemic form from simple, nonsugar starting materials (100).

Sugar Phosphates

In his excellent review of sugar phosphates in 1951, Leloir (101) listed the various methods of phosphorylation that had been introduced up to that time. These included the reaction of acetylated glycosyl halides with "monosilver phosphate," trisilver phosphate, silver diphenylphosphate, or silver dibenzylphosphate for the preparation of glycosyl phosphates, and the use of diphenyl, dibenzyl, or catechol phosphorochloridates for the esterification of alcoholic hydroxyl groups. In addition, the ring opening of sugar anhydrides with salts of phosphoric acid had been used to a limited extent for the introduction of the phosphate moiety. These methods, and especially the use of the dibenzyl and diphenyl phosphorochloridates ($(A_rO)_2POCl$), have been further exploited in the past six years for the synthesis of biologically important sugar phosphates. Moreover, a number of new methods of phosphorylation, aimed particularly at the preparation of cyclo- and polyphosphates, have been introduced.

Glycosyl Phosphates.—From the earlier work in the glucose and galactose series (101) it might have been concluded that reaction of an acetylated glycosyl halide with "monosilver phosphate" or silver dibenzylphosphate, to produce a glycosyl phosphate, proceeds in general with configurational inversion, whereas the corresponding reaction with trisilver phosphate or silver diphenylphosphate proceeds in general without inversion. It is now apparent that the anomeric course of such phosphorylations is governed by somewhat more subtle considerations than simply the nature of the phosphorylating agent.

The reaction of 2,3,5-tri-O-benzoyl-β-D-ribofuranosyl bromide with triethylammonium dibenzylphosphate in benzene solution, followed by removal of protective groups, gives β-D-ribofuranose 1-phosphate in 20 percent yield (102). However, conversion of methyl 5-O-benzyl-α-β-D-ribofuranoside-2,3-carbonate to the corresponding 1-bromide, followed by reaction in benzene solution with triethylammonium dibenzyl-phosphate and subsequent removal of protective groups, gives α-D-ribofuranose 1-phosphate in 60 per cent yield (103). The authors suggest that neighboring group participation of the benzoyl substituent at C-2 in the first instance, and the absence of such effects in the second instance, govern the anomeric course of the phosphorylation.

Treatment of 2,3,4-tri-O-acetyl-α-D-xylopyranosyl bromide with trisilver phosphate yields the α-1-phosphate (104), as would be expected from analogy with the glucose and galactose series. It has now been observed, however, that reaction of the same xylosyl bromide with silver dibenzylphosphate also yields the α-1-phosphate (105). Here, the suggestion has been that made the presence or absence of the hydroxymethyl group at C-5 of the pyranose ring may determine the anomeric course of the reaction with silver dibenzylphosphate, either sterically or through hydrogen bonding effects.

The probability that N-acetyl-D-glucosamine 1-phosphate is involved in mucopolysaccharide synthesis (106) has stimulated interest in the prepara-

tion of this glycosyl phosphate. The α-anomer has been obtained by treatment of the fully acetylated D-glucosaminyl chloride with trisilver phosphate (107). The α-1-phosphate also results from treatment of tri-O-acetyl-D-glucosaminyl bromide with triethylammonium diphenylphosphate, removal of the phenyl groups by hydrogenolysis, and deacetylation with ammonia (108). The final step involves migration of one acetyl group from oxygen to nitrogen.

Monophosphate Esters.—Considerable attention has been focused recently on the preparation of monophosphate esters of the lower-carbon sugars and their derivatives and on the synthesis of pure, individual nucleotides.

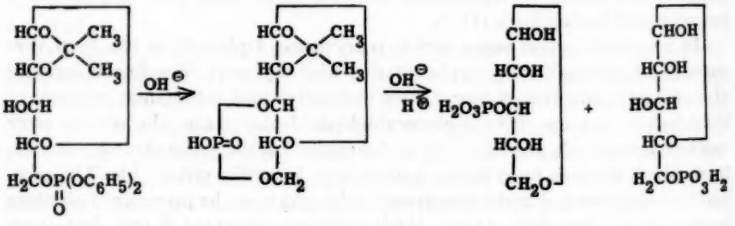
Glycolaldehyde 2-phosphate may be considered as the simplest monophosphate ester related to the sugars. It has been isolated as the amorphous barium salt following periodate oxidation of glycerol 1-phosphate (109). Periodate oxidation of the more complex molecules D-ribose 5-phosphate (110), D-gluconic acid 6-phosphate (111), 3-deoxy-D-*erythro*-2-hexulonic acid 6-phosphate (111), and sphingosine phosphate (112) also produces glycolaldehyde 2-phosphate.

DL-Glyceraldehyde 3-phosphate has been available in pure form for many years (113). However, the synthesis of the pure, biologically-active D-glyceraldehyde 3-phosphate has been accomplished only recently by Ballou & Fischer (114): 1,3:4,6-di-O-methylene-D-mannitol upon benzylation, removal of the methylene groups by acetolysis, and cleavage with periodate provided 2-O-benzyl-D-glyceraldehyde. The latter was converted, through the mercaptal, to the dimethyl acetal. Phosphorylation with diphenyl phosphorochloridate and hydrogenolysis then gave the dimethyl acetal of D-glyceraldehyde 3-phosphate. The latter, which is hydrolyzed autocatalytically to D-glyceraldehyde 3-phosphate in water solution, can be stored conveniently as the crystalline cyclohexylamine salt. New definitive syntheses have also been reported by Ballou & Fischer for D-glyceric acid 2- and 3-phosphates (115) and for dihydroxyacetone 1-phosphate (116). Their D-glyceric acid 2-phosphate shows an optical rotation quite different from that recorded previously for this substance and apparently represents the first pure sample of the compound. By oxidation of the dimethyl acetal of dihydroxyacetone 1-phosphate, followed by hydrolysis, Ballou & Hesse have obtained hydroxypyruvic acid 3-phosphate (117).

In the four-carbon sugar series, D-erythrose 4-phosphate has been prepared (118) through the phosphorylation with diphenyl phosphorochloridate of a suitably substituted D-erythrose derivative and subsequent removal of the blocking groups. Like D-glyceraldehyde 3-phosphate, the tetrose ester can be conveniently stored as the cyclohexylamine salt of its dimethyl acetal, from which the free D-erythrose 4-phosphate is readily obtainable. The ester reacts rapidly with dihydroxyacetone 1-phosphate in the presence of aldolase to give diphosphosedoheptulose. With a cell-free extract of *E. coli*, the tetrose ester condenses rapidly and quantitatively with phosphoenolpyruvate to give 5-dehydroshikimic acid (119), a precursor for the biosynthesis of benzen-

oid amino acids (120). Both of the enantiomorphic erythritol 4-phosphates have also been synthesized by MacDonald, Fischer & Ballou (121) through phosphorylation of appropriately substituted, asymmetric erythritol derivatives. The synthetic D-erythritol 4-phosphate is identical with the ester produced from erythritol and adenosine triphosphate in the presence of *P. pentosaceum* (122).

The preparation of D-xylofuranose 5-phosphate in 15 per cent yield, by phosphorylation of 1,2-O-isopropylidene-D-xylofuranose with phosphoryl chloride, followed by acid hydrolysis, was originally reported by Levene & Raymond (123). The procedure now has been greatly improved through the use of diphenyl phosphorochloridate as the phosphorylating agent (124, 125). Levene & Raymond (123, 126) also recorded several attempts to prepare D-xylose 3-phosphate through phosphorylation of 1,2-O-isopropylidene-D-xylofuranose substituted with various blocking groups at C-5. After acid hydrolysis of the protective groups, however, they invariably obtained D-xylose 5-phosphate, presumably as the result of phosphoryl migration from C-3 to C-5. Recently, Watson & Barnwell (127) suggested that the reverse migration, from C-5 to C-3, might occur when disodium D-xylose 5-phosphate is heated in solution at pH 6.2 and 50° and might thus provide a source of D-xylose 3-phosphate. Their suggestion was based on an observed change in optical rotation and an accompanying appearance of formaldehyde among the products of periodate oxidation on the isomerized mixture. However, Moffatt & Khorana (128) have demonstrated that the isomerization under these conditions involves the formation of D-*threo*-pentulose 5-phosphate from D-xylose 5-phosphate. A similar isomerization of barium D-ribofuranose 5-phosphate to the D-*erythro*-pentulose structure has also been observed (129). Moffatt & Khorana (128) have finally succeeded in preparing the elusive D-xylose 3-phosphate from D-xylose 5-diphenylphosphate (IX). Treatment of the latter with 2*N* NaOH at room temperature converted it to 1,2-O-isopropylidene-D-xylofuranose 3,5-cyclophosphate (X), which was obtained in high yield as the crystalline cyclohexylamine salt. Hydrolysis of the cyclic phosphate with 1*N* NaOH at 100° then provided a mixture of the 1,2-O-isopropylidene-D-xylofuranose 3- and 5-phosphates. After hydrolysis of



IX X XI XII

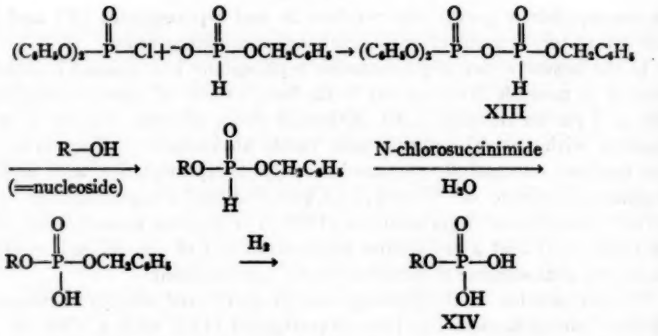
the isopropylidene group, the D-xylose 3- and 5-phosphates (XI and XII) were completely separated by ion-exchange chromatography.

In the hexose series, D-glucosamine 6-phosphate has aroused interest because of its possible involvement in the biosynthesis of mucopolysaccharide (106) and purine riboside (130). Although direct phosphorylation of D-glucosamine with metaphosphoric acid yields an impure product (131), the ester has been successfully prepared through phosphorylation with diphenyl phosphorochloridate of N-acetyl-1,3,4-tri-O-acetyl-β-D-glucosamine (132) and of N-anisylidene-D-glucosamine (133). A crystalline monohydrate of the free ester (132) and a crystalline ammonium salt of the related, synthetic N-acetyl-D-glucosamine 6-phosphate (133) are recorded.

The preparation of the 6-phosphates of acetylated *aldehydo*-hexoses and *aldehydo*-2-deoxyhexoses has been investigated (134) with a view to condensing these with nitrogenous bases to obtain nucleotides. For example, 2,3,4,5-tetra-O-acetyl-D-galactose diethyl mercaptal, upon phosphorylation with diphenyl phosphorochloridate, demercaptalation, and recrystallization of the product from methanol, yielded a crystalline monomethanolate of 2,3,4,5-tetra-O-acetyl-D-galactose 6-diphenylphosphate. The corresponding derivatives of 2-deoxy-D-galactose and 2-deoxy-D-glucose were obtained as sirups.

A minor alteration in the structure of the simple sugar phosphates ($R\text{-}OPO_3H_2$) is represented by the sugar phosphonates ($R\text{-}PO_3H_2$), and studies of the preparation and biochemical behavior of the latter have been reported. Treatment of epiiodohydrin (135) or epibromohydrin (136) with triethyl phosphite at 135 to 140°, followed by hydrolysis of the resulting diethyl 2,3-epoxypropylphosphonate, gives 2,3-dihydroxypropylphosphonate, the deoxy analogue of the biochemically important glycerol 1-phosphate. Similarly (137), reaction between 1,2,3,4-tetra-O-acetyl-6-bromo-6-deoxy-D-glucose or benzyl 2,3,4-tri-O-acetyl-6-bromo-6-deoxy-D-glucoside and ethyl diphenyl phosphite, followed by removal of protective groups, gives 6-deoxy-D-glucose 6-phosphonate, the deoxy analogue of D-glucose 6-phosphate.

Todd and his associates, who have made the principal advances in the synthesis of nucleotides, use dibenzyl phosphorochloridate extensively for monophosphorylation in this field. One example is the phosphorylation of 5′-O-trityladenosine (138) to give, after removal of protective groups, adenosine 2′- and 3′-phosphates identical with the adenylic acids *a* and *b* from natural sources (139). Certain secondary hydroxyl groups in the nucleoside structure, however, do not phosphorylate readily with dibenzyl phosphorochloridate. For such instances, the Todd group has developed a valuable alternative method using "O-benzylphosphorus O,O-diphenylphosphoric" anhydride (140). This mixed anhydride (XIII), which is obtained in crude form by the reaction of diphenyl phosphorochloridate and monobenzylphosphite, reacts readily with the alcoholic hydroxyl group to give a nucleoside monobenzyl phosphite. The latter is oxidized, through chlorination with N-



chlorosuccinimide followed by mild hydrolysis, to the nucleoside benzylphosphate. Hydrogenolysis then gives the desired nucleotide (XIV). The "mixed anhydride" method has been used for the definitive synthesis of adenosine 2′-phosphate (adenylic acid *a*) from 3′,5′-di-O-acetyladenosine (141). 5′-O-Acetyldeoxyadenosine, which does not phosphorylate satisfactorily with dibenzyl phosphorochloridate, also has been phosphorylated by the mixed anhydride method (142).

Tetra-*p*-nitrophenylpyrophosphate, prepared from di-*p*-nitrophenyl phosphoric acid and dicyclohexylcarbodiimide ($C_6H_{11}N{=}C{=}NC_6H_{11}$) (143), is reported to be a powerful phosphorylating reagent. Thus, 2′,3′-O-isopropylideneguanosine has been converted in high yield to the 5′-phosphate with this reagent (144).

Dinucleoside phosphates (R—O—P—O—R′) and dinucleotides (R—O—P—O—R′—O—P), obtained from the degradation of nucleic acids, should provide important information concerning the fine structure of these polymers. Accordingly, the synthesis of such degradation products by unequivocal routes is of especial interest. Gulland & Smith (145) obtained the first synthetic, symmetrical dinucleoside phosphate by condensing 2′,3′-O-benzylideneuridine with phenyl phosphorodichloridate ($C_6H_5O—POCl_2$) and removing the protective groups. The product which they believed to be bis(uridine) 2′,2′-phosphate, is now known to be the 5′5′-phosphate. A nonsymmetrical dinucleoside phosphate was first obtained by Elmore & Todd (146). This synthesis depended in part upon the observation that a nucleoside dibenzylphosphate can be readily monodebenzylated by a variety of reagents including tertiary bases (147), lithium chloride (148), phenol (149, 150, 151), and barium or sodium iodide (152). Thus, Elmore & Todd phosphorylated 2′,3′-O-isopropylideneadenosine with dibenzyl phosphorochloridate, monodebenzylated the product, and treated the silver 2′,3′-isopropylideneadenosine 5′-benzylphosphate with 5′-deoxy-5′-iodo-2′,3′-isopropylideneuridine to obtain, after removal of protective groups, adenosine-5′ uridine-5′ phosphate. An alternative method for preparing nonsymmetrical dinucleoside phosphates and dinucleotides was suggested by the work of Baer & Kates (153). These authors, working in the glyceride

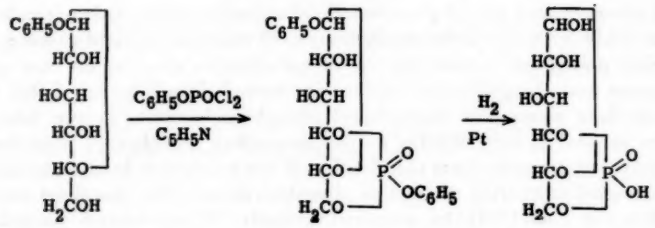
field, observed that phenyl phosphorodichloridate could be made to condense successively with two different hydroxylated reagents to yield a non-symmetrical phosphoric diester, by use of quinoline to catalyze the first condensation and of pyridine to catalyze the second. It will be noted that the intermediate monoalkyl monophenyl phosphorochloridate in the Baer & Kates method is very similar to the monoalkyl monobenzyl phosphorochloridate that results from chlorination of the nucleoside benzyl phosphite in the mixed anhydride method of phosphorylation (140) described above. Michelson & Todd (154) thus prepared thymidine-3′ thymidine-5′ phosphate by condensing crude 5′-O-acetylthymidine 3′-(benzyl phosphorochloridate) (obtained by the mixed anhydride method) with 3′-O-acetylthymidine and removing the protective groups. They also obtained the first synthetic dinucleotide in similar fashion through the condensation of 3′-O-acetylthymidine and thymidine 3′-(benzyl phosphorochloridate) 5′-dibenzyl phosphate. The product, which they represent symbolically as T5′-P-3′T5′P, behaved enzymatically in all respects like the polynucleotide fragments obtained in the degradation of deoxyribonucleic acid. Strong confirmation for the 3′,5′-internucleotide linkage in the polymer is thus provided. More recently, a nonsymmetrical diribonucleoside phosphate (adenosine-2′ uridine-5′ phosphate) has been obtained by a similar series of reactions (155).

Cyclophosphate Esters.—Interest in the cyclophosphate esters of the sugars centers around their role as intermediates both in phosphate group migration and in the degradation of ribonucleic acids. The detection by Markham & Smith of ribonucleoside cyclophosphates in the ribonuclease-(156) and alkali-(157) catalyzed hydrolysates of ribonucleic acid has aided greatly in the gradual elucidation of the mechanism (158, 159, 160), and consequent ease, of the hydrolysis of this polymer as compared with deoxyribonucleic acid.

A cyclophosphate sugar ester apparently was obtained as early as 1914 by Emil Fischer (161) through treatment of D-glucopyranosyltheophylline with phosphoryl chloride in pyridine. The product is now believed to be the 4′,6′-cyclophosphate (162). Similarly, Forrest & Todd (163) have observed that riboflavin, when treated with phosphoryl chloride in moist pyridine, gives the corresponding 4′5′-cyclophosphate. The yields of the cyclic esters obtainable by such direct phosphorylations are considerably improved through the use of phenyl phosphorodichloridate, rather than phosphoryl chloride, and pyridine (162). Thus phenyl β-D-glucopyranoside (XV) has been converted in good yield to D-glucose 4,6-cyclophosphate (XVI).

Noncyclic sugar phosphates containing a free hydroxyl group α or β to the ester grouping are readily converted, respectively, to the 5- or 6-membered cyclophosphates through treatment with alkali (128, 157), trifluoroacetic anhydride (164, 165), or dicyclohexylcarbodiimide ($C_6H_{11}N{=}C{=}NC_6H_{11}$) (166). Direct treatment of ribonucleic acid with potassium *t*-butoxide in *t*-butanol and formamide provides high yields of the nucleoside 2′,3′-cyclophosphates (167).

Pyrophosphate linkages that lie adjacent to free hydroxyl groups also are



XV XVI

readily cleaved by mild alkali to produce cyclophosphates, as in the formation of riboflavin 4′,5′-cyclophosphate from "flavin-adenine dinucleotide" (163), or D-glucose 1,2-cyclophosphate from "uridine-diphosphate-glucose" (168), and of a cyclophosphate of pantetheine from coenzyme A (CoA) (169).

Polyphosphate Esters.—The occurrence of di- and triphosphate linkages in such coenzymes as adenosine di- and triphosphate (ADP and ATP), di- and triphosphopyridine nucleotide (DPN and TPN), flavin adenine dinucleotide (FAD), CoA and uridine-diphosphate-glucose (UDPG) makes the synthesis of such polyphosphates of theoretical and practical importance. Some of the methods of direct phosphorylation described above have been used to synthesize multiple phosphate linkages and, in addition, a number of interesting new methods have been introduced.

In the first paper of the extensive series on nucleotide synthesis by Todd and his associates, Baddiley & Todd (170) described the synthesis of ADP by interaction of dibenzyl phosphorochloridate and silver adenosine 5′-benzylphosphate, the latter having been obtained by the partial, acid hydrolysis of 2′,3′-O-isopropylideneadenosine 5′-dibenzylphosphate. The intermediate adenosine 5′-tribenzylpyrophosphate, which yielded ADP on hydrogenolysis, was subsequently monodebenzylated (171), and the product phosphorylated again with dibenzyl phosphorochloridate to give, after hydrogenolysis, ATP. Surprisingly, reaction of disilver adenosine 5′-phosphate with two molecular equivalents of dibenzyl phosphorochloridate, followed by hydrogenolysis, also gives the normal ATP (172), presumably through rearrangement via the trimetaphosphate of the expected, branched triphosphate.

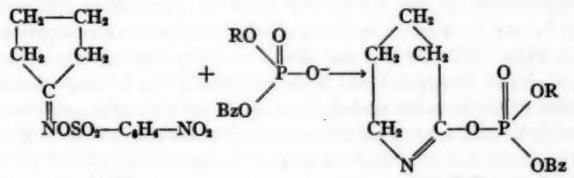
Formation of the pyrophosphate linkage by direct coupling of monophosphates under the influence of dicyclohexylcarbodiimide (DCC) or trifluoroacetic anhydride has been utilized in the synthesis of *bis*(adenosine) 5′-pyrophosphate and *bis*(uridine) 5′-pyrophosphate (173). Mixed pyrophosphates may also be obtained by this method. For example, treatment of the mixed pyridine salts of uridine 5′-phosphate and α-D-glucose 1-phosphate with DCC gives a complex mixture containing about 40 per cent of UDPG (174). Moreover, phosphoric acid may be directly coupled with nucleoside monophosphates, through the agency of DCC, to provide the corresponding poly-

phosphates. In this way adenosine and uridine 5′-phosphates yield, respectively, ADP plus ATP (175) and UDP plus UTP (176).

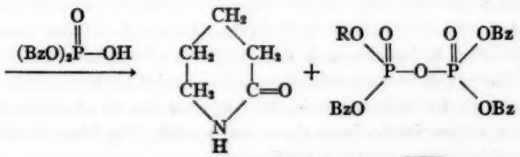
Nucleoside monobenzyl phosphorochloridates, which are obtained as intermediates in the "mixed anhydride" method of phosphorylation described above, may be condensed with salts of nucleotides to produce the pyrophosphate linkage. Thus, 2′,3′-O-isopropylideneadenosine 5′-(benzyl phosphorochloridate) condenses with monothallous riboflavin 5′-phosphate to provide, after removal of proective groups, P^1-adenosine-5′ P^2-riboflavin-5′ pyrophosphate (FAD) (149). Similarly, 2′,3′-di-O-acetyl- or 2′,3′-O-isopropylideneuridine 5′-(benzyl phosphorochloridate) condenses with triethylammonium tribenzylpyrophosphate to give, after removal of protective groups, UTP (150). UDPG and UDPGal also have been synthesized by this method through condensation of 2′,3′-di-O-benzyluridine 5′-(benzyl phosphorochloridate) with the tri-*n*-octylammonium hydrogen salts of the appropriate hexose 1-phosphates (177).

An "exchange method" of preparing dinucleoside pyrophosphates was suggested by the observation of Corby, Kenner & Todd (178) that treatment of a mixture of tetraphenylpyrophosphate and dibenzyl phosphoric acid with base yielded tetrabenzylpyrophosphate and diphenyl phosphoric acid. The reaction apparently proceeds to form the most stable anhydride (of the weaker acid) and the most stable ion (of the stronger acid). The method, however, has not given encouraging results when applied to the synthesis of nucleoside pyrophosphates (173).

Imidoyl phosphates, which are obtainable from the Beckmann rearrangement of ketoxime arylsulfonates in the presence of substituted phosphate anions, also have been utilized in the synthesis of polyphosphate linkages (179). For example (180), cyclopentanone oxime *p*-nitrobenzenesulfonate (XVII) reacts with tetra-*n*-butylammonium 2′,3′-O-isopropylideneuridine 5′-benzylphosphate to give the imidoyl phosphate (XVIII, R=2′,3′-O-isopropylideneuridine 5′-). Phosphorolysis of the latter with dibenzyl phosphoric acid then gives 2′,3′-O-isopropylideneuridine 5′-tribenzylpyrophos-



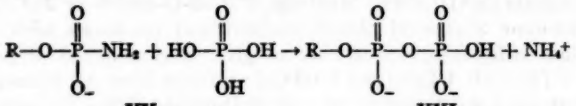
XVII XVIII



XIX

phate (XIX). Removal of the protective groups from XIX gives uridine 5′-pyrophosphate in 40 per cent yield.

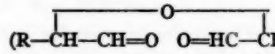
Salts of phosphoramidic acids condense directly with phosphates or phosphoric acid, at room temperature in formamide solution, to produce polyphosphates (181). Thus, adenosine 5′-phosphoramidic acid (XX, R=adenosine 5′—) condenses with phosphoric acid to give 35 per cent of ADP (XXI). Similarly, adenosine 5′-phosphate and ten equivalents of monotriethyl-ammonium phosphoramidic acid (XX, R=H) gives a mixture



XX XXI

containing 24 per cent of AMP, 27 per cent of ADP, 21 per cent of ATP, and 17 per cent of higher adenosine polyphosphates.

Miscellaneous.—The dialdehydes resulting from the periodate oxidation of ribonucleoside 5′-phosphates



undergo a smooth β-elimination of the phosphate moiety under mild alkaline conditions (pH 10 to 10.5) that are without effect on intact ribonucleic acid. The reaction, which has been applied to di- (182) and trinucleotides (183), and to the dihydrogen and benzyl hydrogen 5′-phosphates of adenosine (184), is a potentially valuable tool for the stepwise degradation of polyribonucleotides.

Uridine 3′-dimethylphosphate and 3′-dibenzylphosphate have been synthesized and found to be unstable in water over the entire pH range (185). It is therefore concluded that the existence of phosphate triester linkages in ribonucleic acid is unlikely.

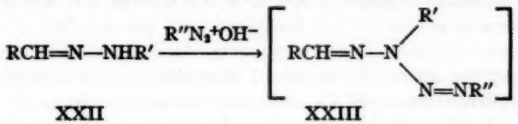
Hydrogenation of the 4,5-double bond in pyrimidine nucleotides has long been known to render the N-glycosidic linkage more susceptible to acid hydrolysis (186, 187). By the use of a new 5 per cent rhodium-on-alumina catalyst, Cohn & Doherty (188) have performed the hydrogenation on the uridylic and cytidylic acids under particularly mild conditions. The resulting dihydrouridylic acid may then be converted by mild alkali at room temperature to the N-D-ribosyl phosphate of β-ureidopropionic acid. The latter, in turn, in hydrolyzed by dilute acid at room temperature to D-ribose phosphate and β-ureidopropionic acid. In this reaction sequence, uridylic acids *a* and *b* give, respectively, D-ribose 2- and 3-phosphates. A similar result had been recorded previously by Baron & Brown (189) who degraded the pyrimidine moiety of the cytidylic and uridylic acids *a* and *b* by treatment with hydrazine, rather than by hydrogenation and hydrolysis, to obtain D-ribose 2- and 3-phosphate, respectively, from the *a* and *b* acids. The identity of the pyrimidine nucleotide isomers is thus confirmed.

Mannoheptulose phosphate has been found to accompany sedoheptulose phosphate and other intermediates of carbohydrate synthesis in avocado leaves (190).

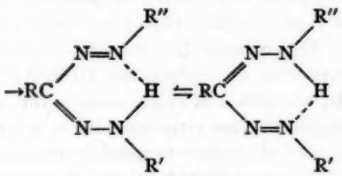
The Formazan Reaction

The presence or absence of ring structures in the various sugar hydrazones and osazones, and the nature of such rings when present, is a problem of long standing (191). The formazan reaction, recently introduced into the sugar field by the late Professor G. Zemplén and his associate L. Mester (192), is proving to be a diagnostic tool of great value in the continuing attack on this structural problem.

The formazan reaction consists of the coupling of a diazonium compound, in pyridine or alkaline ethanol solution, with an acyclic aldose phenylhydrazone derived from a primary hydrazine. To proceed, the coupling reaction requires not only the presence of a true Schiff base system (—CH=N—), but also the presence of an imino hydrogen atom (=N—NH—) in the hydrazone (XXII), since reaction leads first to a diazohydrazone (XXIII) that rearranges to the formazan (XXIV) (193, 194). The chelate equilibrium structure for the sugar formazans has been confirmed by the observation that D-galactose *p*-bromophenylhydrazone and diazotized aniline yield the same



XXII XXIII

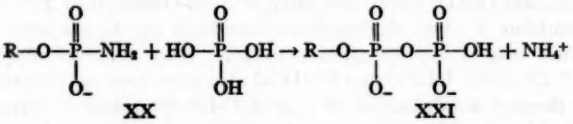


XXIV

formazan as do D-galactose phenylhydrazone and diazotized *p*-bromoaniline (195). Hydrazones that do not fulfill both of the above requirements, and hence give a negative formazan reaction, include acyclic ketose hydrazones, acyclic aldose hydrazones derived from asymmetric, secondary hydrazines ($RCH{=}N{-}NR'R''$), and all derivatives in which the hydrazone function is involved in ring formation. For example, D-glucose "α"-phenylhydrazone of known ring structure (196) and D-galactose 1-methyl-1-phenylhydrazone do not react, whereas D-galactose phenylhydrazone of known acyclic structure (197) provides the formazan in 86 per cent yield (192, 195). With sugar osazones, a positive reaction indicates the presence of the structure —CH =N—NHR at C-1. The formazans derived from sugar hydrazones are brown to red in color, and give a blue coloration with sulfuric acid, while those from osazones are violet to black. Both classes are insoluble in water.

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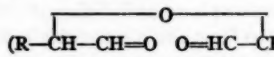
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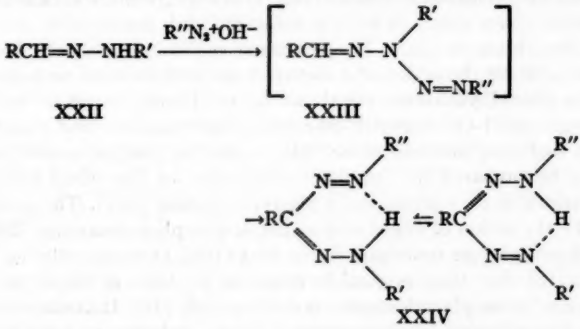
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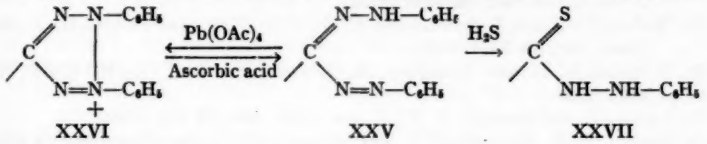
XXII XXIII XXIV

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In recent studies, the formazan reaction has been applied to a variety of structural problems in the hydrazone and osazone series. Mester & Major (198) have observed that the ability to form formazans correlates closely with the mutarotation behavior of sugar hydrazones. For example, as the mutarotation of D-galactose phenylhydrazone in pyridine progresses, the yield of formazan obtainable from the solution falls regularly from over 80 per cent to about 20 per cent. Similar studies were made with the phenylhydrazones of D-mannose and L-rhamnose. Of the three D-glucose phenylhydrazones, Skraup's hydrazone (199) and the "α"-hydrazone (200) do not react, whereas the "β"-hydrazone (201) provides a formazan in yields dropping from 60 per cent to zero as mutarotation progresses. Since the formazan test is carried out in organic solvents, it can also be applied to the acetylated sugar hydrazones. Thus, the D-mannose anhydrophenylhydrazone obtained by pyridine-acetic anhydride acetylation of D-mannose phenylhydrazone was found to give a negative test, in agreement with the pyrazoline structure proposed for this substance by Wolfrom & Blair (202). D-Glucose phenylosazone yields a formazan in alkaline ethanol but not in pyridine (203). The reaction presumably fails in the latter solvent because of the chelate ring structure (204) of the osazone. Acetylation of the D-glucose phenylosazone formazan introduces four O-acetyl groups, indicating a completely open-chain structure for the formazan and, presumably, also for D-glucose phenylosazone (205). The two osazones ("mixed osazones A and B") obtained (206) by the action of 1-methyl-1-phenylhydrazine on D-glucose or D-fructose phenylhydrazones are shown by the formazan test to be, respectively, D-glucose 1-(1′-methyl-1′-phenyl)-2-phenylosazone and a mixture of this with D-glucose phenylosazone (203). D-glucose phenylosazone formazan also may be prepared by the successive action of diazotized aniline and phenylhydrazine on D-glucosone 1-phenylhydrazone (207). The anhydrides formed by the action of dilute acid on the hexose phenylosazones (208) have been assigned various structures in the past (191). Only recently has it been demonstrated that their original formulation by Diels & Meyer as normal 3,6-anhydrohexose phenylosazones is correct (209, 210). In confirmation, the 3,6-anhydro-D-glucose phenylosazone ("Diels' anhydro-osazone") readily yields a formazan derivative (211). The formazans of 1,2-O-isopropylidene-5-aldo-D-xylose phenylhydrazone and *xylo*-trihydroxyglutaric dialdehyde *bis*-phenylhydrazone have been prepared (212).

The formazan reaction has also been applied to a number of oxidized polysaccharides. The phenylhydrazone of periodate-oxidized starch, which contains somewhat more than one phenylhydrazine residue for each dialdehyde group (213), gives a positive formazan reaction, thus indicating an open-chain structure for the hydrazone moiety (214). Similar results were obtained with periodate-oxidized cellulose, xylan, inulin, and dextrin. Cellulose oxidized with nitrogen dioxide yields principally poly-D-glucuronic acid (215). The presence in the product of aldehyde groups (which could result from hydrolytic degradation) is indicated by a positive, qualitative formazan reaction (216).

The colored sugar formazans (XXV) are readily oxidized by lead tetraacetate or N-bromosuccinimide to the colorless tetrazolium derivatives (XXVI) (217, 218). Like 2,3,5-triphenyl-tetrazolium chloride (TTC), the latter are of considerable interest as biological indicators in living systems



XXVI XXV XXVII

and have the added advantage of being less toxic than TTC (217). Reduction of the tetrazolium derivatives with ascorbic acid reforms the formazans. Reduction of the formazans with hydrogen sulfide leads to the corresponding hydrazides of thioaldonic acids (XXVII) (219, 220). The phenylhydrazide of D-galactothionic acid thus obtained shows significant tuberculostatic activity *in vitro* and *in vivo* (219). The formazans derived from sugar hydrazones can be converted in good yield to the corresponding aldonic acids by treatment with nitrous acid (217). Use of the formazans in the identification of aldoses has been suggested (220).

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THE BIOCHEMISTRY OF MUSCLE[1,2]

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Symposia, Reviews, Monographs

The great interest of biologists in muscular activity and its chemical and physicochemical bases has led to the publication of about 500 chemical and physicochemical investigations since Mommaerts' review (1) and of a number of communications which have studied exclusively the mechanical and physical behaviour of living muscles. Some of the papers that are of topical interest may be cited here (2 to 6).

The great interest in muscle has moreover led to comprehensive treatment in symposia, monographs, and reviews. The *Proceedings of the 3rd Medical Conference on Muscular Dystrophy* (7) contain a very thorough survey of the problems of muscle and of the clinical questions involved. The physiology and biochemistry of muscle (without clinical aspects) are given in five lectures on "Fibrous Proteins and Their Biological Significance," printed in the *Symposia of the Society for Experimental Biology* (8 to 12). The accounts given by many experts in the *British Medical Bulletin* (13) extensively cover the "Physiology of Voluntary Muscle." Excellent reviews by Perry (14) and Feigen (15) must be mentioned, and a monograph by Dubuisson (16).

Further reviews deal with certain branches of the physiology and biochemistry of the muscle. Reviews on fish protein have been written by Hamoir (17, 18), and on structural and contractile proteins by Bailey (19), H. H. Weber (20), Hamoir (21), and A. Weber & Hasselbach (22), while a monograph by Szent-Györgyi (23) deals only with L-myosin. Whether ATP splitting or ATP binding is the cause of contraction has been discussed in a review by Morales and co-workers (24). In his excellent review Wilkie (25) has used our knowledge of muscle energetics to examine critically many theories on contraction. With special reference to the data found by Hill

[1] The survey of the literature pertaining to this review was completed in October, 1956.

[2] The following abbreviations and symbols are used in this chapter: ADP for adenosinediphosphate; AM for actomyosin (Actin plus L-myosin); AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; ATPP for adenosinetetraphosphate; ATPPP for adenosine pentaphosphate; CP for creatine phosphate; CTP for cytidine triphosphate; DNP for paradinitrophenol; DPN for diphosphopyridine nucleotide; EDTA for ethylendiaminotetraacetate; GTP for guanosine triphosphate; IDP for inosine diphosphate; IP for isoelectric point; ITP for inosine triphosphate; mysin for mixture of actomyosin and L-myosin; NTP for nucleoside triphosphate; PP for pyrophosphate; PPP for triphosphate; SNC for thiocyanate; TPN for triphosphopyridine nucleotide; and UTP for uridine triphosphate.

[3] Translated by Marga Weber.

and his school A. Huxley (26) has evaluated the most recent and important results concerning muscular fine structure and its behaviour on undergoing stimulation and contraction. Fleckenstein (27) proposes that muscle contraction is related energetically to the shifting of ions accompanying excitation.

New Methods

Enzymatic methods.—The enzymatic methods for determining the quantities of energy-rich organic phosphates have been considerably improved from 1954 to 1956. The application of these methods is facilitated by excellent descriptions of the isolation of pure enzymes by means of simple methods, e.g., from one aqueous extract of rabbit musculature the following enzymes can be prepared in a highly purified state: aldolase, α-glycerophosphate dehydrogenase, pyruvate kinase, and phosphoglyceraldehyde dehydrogenase (28). This applies similarly to creatine phosphokinase, hexokinase, and myokinase (29). Phosphoglyceric kinase can be prepared from pea-meal (30).

The known determination of ATP, ADP, and AMP, by the reduction of DPN or TPN is developed to such an extent today that changes in concentration can be determined in a single twitch (29 to 33). Chappell & Perry (29) designed a procedure for determining ADP or ATP in extremely low concentrations if only one of the two substances is present; if they are both present only their sum can be determined. The process is a phosphate transfer from CP to ADP, forming ATP, and finally to glucose under conditions that make the amount of ADP or ATP a rate-limiting factor. The required enzymes are creatine phosphate kinase and hexokinase. If myokinase is added, AMP can be determined according to the same method.

ATP in the presence of other labile phosphate compounds can be used for phosphorylating phosphoglyceric acid with the aid of phosphoglyceric acid kinase, and after reaction with hydroxylamine hydrochloride it can be determined colorimetrically as Fe-complex (30) [for ATP determination in ATP-ADP mixtures, cf. also (34)].

ATP, the purine ring of which contains C^{14}, can be obtained from C^{14}-acetate by means of *Torula* yeast (35). With the aid of enzymatic systems it is possible to incorporate P^{32} into definite sites of the triphosphate of ATP (36).

Chromatography, and paper electrophoresis.—Procedures for separating ATP, ADP, AMP, UTP, CTP, GTP, and ATPP, with the help of Dowex resins have been developed (37 to 40). It is recommended that paper chromatography be combined with the adsorption of nucleotides by charcoal (41). Keto acids, such as pyruvic acid and ketoglutaric acid, can be determined by paper chromatography (42). Paper electrophoresis seems to be suitable for separating crystalloids such as adenosinephosphates (43), CP, creatine, β-alanine, peptides, and nucleotides (44). It is reported that paper electrophoresis of muscle proteins gives the same mobilities as those obtained with cataphoresis according to the Tiselius method (45 to 47).

Miscellaneous chemical observations.—When phosphate is determined according to Fiske & Subbarow the values found are too low if ATP, ITP, or ADP, are present in concentrations $>7\times10^{-3}M$. In this case the solution must be diluted before colorimetry is applied (48). A quantitative precipitation of PP by Mg is possible only in the absence of adenosinephosphates (49).

The determination of glycogen by using anthrone reagent has been further developed (50). The activity of redox enzymes, e.g., succinic dehydrogenase, can be determined by the reduction of tetrazolium salts (51).

For the determination of SH groups in proteins a modification of Barnett & Seligmann's method has been presented (52).

Methods concerning the living muscle.—The extremely important fixation of muscles and other tissues in a definite state of function appears to be extremely successful when using the method described by Mommaerts and coworkers in 1955 (53). Isometric tensions between 50 mg. and 2 mg. can be measured by means of a triode (54), just as the phase-plane method can be applied for studying mechanical muscle properties (55).

Of particular importance in biochemical research is the development of the interference microscope with a high resolving capacity (56), which, besides its optical advantages, also offers the highly important possibility of determining quantitatively the protein concentrations of special cell areas (e.g., of the A and I bands) in the living cell.

Recently Discovered Crystalloids, New Data on Known Crystalloids, and Trace Substances in Muscle

New phosphate compounds.—From a biological point of view the most important fact appears to be the discovery of further nucleoside-polyphosphates. Thus Bergkvist & Deutsch have isolated UTP (57) and GTP (58) from muscle and have identified CTP in muscle (58). CTP is shown to be in numerous other tissues (59). In addition to the other adenosine phosphates, ATPP has been found in muscle (40, 60, 61); traces of ATPPP even appear to be present in yeast (62). The Szent-Györgyi school has isolated from muscles an isomer of IDP, which in contrast with normal IDP fluoresces in the presence of Ca or Zn (63, 64).

In worms phosphagens, such as taurocyamine, and glycocyamine phosphate are found in addition to CP and arginine phosphate (65, 66). Phosphodiesters of L-serine and ethanolamine have been identified in ample quantities in turtle muscle (67).

New data on known crystalloids.—Szent-Györgyi's view (64) that ATP in the presence of alkaline earths forms a quadridentate gelate has been substantiated by observations on the rotary dispersion (68). The extremely high affinity constants of the complex formation between adenosine phosphates and PP, on the one hand, and alkaline earths, on the other, show that polyphosphates in the presence of physiological concentrations of alkaline earths are, for the most part, alkaline earth complexes (69, 70). These affinity con-

stants are between about 10^3 and 10^4 (69). Small shifts of the electrotitration curve are ascribed to a slight complex formation with K or Na (71, 72).

Very careful investigations of the ultraviolet absorption of NTP, NDP, and NMP show that the pK values for the 6-NH_2 groups lie always between 4.8 and 3.8 and those for the 2-NH_2 group at about 3. In case an enol group is in the 6-position the apparent pK is about 9.5 (73, cf. also 74).

New thermodynamic data.—The high affinity of phosphate compounds for alkaline earths must be considered in thermodynamic calculations, because this affinity is often different for the substrate and for the reaction products. This is the case with Mg salts of glucose-1-phosphoric acid, glycerol-2-phosphoric acid, and phosphoric acid (75). Naturally, the thermodynamic effects that are due to the neutralization of H ions must be considered, provided that these are liberated during the fermentative process. Data on the ionization of tris buffers and phosphate buffers are now available (76).

The enthalpy change during the hydrolysis of ATP into ADP+P+H^+ is about 5 kcal. per mole after deducting the neutralisation heat of H^+ (77, 78, 79). The heat liberated corresponds well with the fact that the ΔF of ATP hydrolysis is 4.3 kcal. greater than the ΔF of glutamine (80). Hence, there is a ΔF of ATP hydrolysis of 7.5 kcal. to 8 kcal. (77, 80, 80a). The value 7.5 kcal. is concluded from the determination of the equilibrium of the hexokinase reaction (80b).

These thermodynamic values correspond rather satisfactorily with the ΔH values found for the hydrolysis of other polyphosphates: the value for pyrophosphate formerly found by Ohlmeyer & Shatas is lowered to 7.3 kcal. (81), while the ΔH of hydrolysis of hexametaphosphate is given as 9.7 kcal. (82). These values are much lower than the ΔH and ΔF values given by Meyerhof and co-workers for the hydrolysis of the energy-rich P∼O∼P bonds of trimetaphosphate (83).

Trace substances and heavy metals.—Heart and skeletal muscles contain no beryllium, only a few hundredths of parts per million of Co, B, and Hg (84), but a few mg. per cent of Al (85). It is reported that the Fe content of different muscles varies between 0.5 and 10 mg. per cent (86). The extracellular space has been determined only for the skeletal muscle. It is between 10 and 25 per cent according to the kind of muscle (87 to 93).

Membrane

Ion exchange.—A large number of papers concern the problem of how the Na pump affects both the membrane potential (98) and the energy requirement (95, 96, 97) of the muscle. Isotope exchange methods (94 to 97) or microinjections of solutions of NaCl and KCl into the muscle fiber have been used (98). The energy requirement seems to be substantially higher for mammals (96) than for frogs (97).

Potassium extrusion is lowered by Ca without correspondingly affecting the resting potential, so that a competition is assumed between K and Ca

for the membrane colloids entailing a genuine change in the membrane potential by Ca but not an effect on an ion pump (99).

Several papers find that a part of the muscle Na is less easily exchangeable, and it is therefore presumed to be bound to protein (100, 101, 102). It is reported that Na extrusion from the muscle cell is accelerated by a stretching of the muscle by 15 to 40 per cent of the standard length (103).

For biochemistry, the data for the pH inside the fiber are of special importance. With the aid of an intracellular electrode a pH of about 7 inside crab muscles has been determined (104, 105). Conway's calculation of an inside pH of approximately 6 based on the pH measurement outside and on the application of the Donnan equilibrium of the K ions is subjected to a careful criticism by A. V. Hill. Hill comes to the conclusion that the distribution of H^+ is independent of the Donnan proportion of K^+ (106). Thus, the former direct measurements made by Meyerhof, Furusawa & Kerridge, and by the Michaelis school, on muscle brei achieve a new validity (106). Their result of a pH of about 7 is of the same value as that measured with the intracellular electrode.

It is claimed that the ion exchange of the excited membrane is associated with small, rapid decreases in muscle volume which take place immediately after stimulation (107, 108).

Membrane activity and its effect of muscle contraction.—In connection with the basic work done by Hill & MacPherson (109) it has been shown that the activity of the muscle can be influenced by those substances that certainly do not penetrate into the fiber during the course of the experiment. Both the power and the heat production of the single twitch of the muscle fiber are promoted by the anions, Br^-, I^- (109), and NO_3^- (109, 110, 111), and by the substances, tetraethyl ammonium ion, tetramethyl ammonium ion, tetramethyl choline (112, 113, 114), quinine, and quinidine (115), and caffeine (111). However, the single twitch is not intensified by ouabaine, Ca (112), tubocurarine, or eserine (115). Tetrabutyl ammonium ion diminishes the twitch (112). In all these cases it is the duration of the active state that is influenced, not its intensity. This has been shown by mechanical, and occasionally by thermoelectrical, measurements (104 to 115, 120) of the duration of the active state of the contractile structure. The so-called staircase phenomenon of a series of single twitches is likewise explicable by the prolongation of the active state (116). The normal duration of the active state of frog muscle at 0°C. is 25 msec. (117, cf. also 118). As to whether the prolongation of the active state is associated with a prolongation of the action potential (112, 113, 114), or not (109), is still to be decided.

Under appropriate circumstances, a single stimulus can be transformed into a series of excitations by nitrate ions (110). Spontaneous series of discharges arising as a consequence of Ca deficiency become more frequent at a a definite degree of stretch (119).

The question concerning the degree to which the ions of the extracellular

space are bound to the membrane has been examined by measuring their cataphoretic mobility at the muscualr surface. Na, K, and phosphate are almost as movable as in free solution (121). Analogous investigations with the above-mentioned specifically acting ions have not yet been made.

It is important that the muscle membrane is able to control the active state of the contractile structure even for some time after the stimulation; for in this case it is possible that the release of Hill's extra-energy can be controlled by the membrane.

Of great importance is the discovery of A. Huxley and co-workers that the excitation of the membrane can be transmitted to the contractile structure only from well-defined closely circumscribed areas of the sarcolemma. These areas are always located on the I band of the fibrils; but their localization differs slightly for different muscles. Furthermore, in the areas concerned, only definite spaces of the circumference transmit the excitation inwards (25, 122, 123).

Granules, Sarcosomes

Oxidation and phosphorylation.—It now seems to be well established for the muscle that oxidative phosphorylation occurs only in the granules (14, 124, 136a). The P/O ratio found in the mitochondria of the muscle is about the same as that found in the mitochondria of other organs (125, 126, 133). For the oxidation of ketoglutaric acid it is 3 (125) or >3 (126); for that of glutamate, fumarate, malate, β-oxybutyrate, a little lower (126); and for that of succinate about 2 (126).

Consequently, enzymes of the tricarboxylic acid cycle have been prepared from muscles in more or less highly purified form, namely, isocitric dehydrogenase (127) and succinic dehydrogenase (128). The oxidation of cytochrome is combined with ATP phosphorylation in the same way as in liver mitochondria (129, 130). The dehydration of α-ketaglutorate to succinyl CoA leads to succinate without forming ATP, provided that the enzyme succinyl CoA deacylase alone is present. However, in the presence of a phosphorylation system+ADP, ATP is formed as well as succinate (131). The muscles of animals that are poisoned with 3,5-dinitroorthocresol lose the majority of their CP and a great part of their ATP (132).

Adenosinetriphosphatase, myokinase-, deaminase-activity in the granules of muscle.—Like the granules of other organs, muscle granules show adenosinetriphosphatase activity (133 to 138), myokinase activity, and AMP-deaminase activity (14, 135). It is reported that these activities of muscle granules are often higher than the corresponding activities of liver mitochondria (135, 136). The adenosinetriphosphatase activity of the muscle granules is also latent as long as the granules are undamaged (133, 134); it becomes manifest in the presence of DNP (133, 136, 137). It is reported that the adenosinetriphosphatase of granules splits nucleosidephosphates in the following sequence of rates: ATP>ITP>IDP>ADP (139).

The microsomes that are observed in addition to the large sarcosomes in

muscle extracts are chemically very similar to the microsomes of other organs (135). The large sarcosomes lying between the muscle fibrils in characteristic arrangement (14, 140) are described as rodlets, which behave as perfect osmometers as long as they are undamaged (134).

Relaxing factor.—The relaxing factor discovered by Marsh (141) is a protein also localized in the granules of the muscle. Contracted isolated fibrils are induced to relax by irrigating them with granular suspensions. Of more importance, however, is the fact that it is possible to spin down completely at 35,000 g, and incompletely at 18,000 g, the relaxing factor of crude muscle extractions. The entire activity of the relaxing factor is then to be found in the suspension of the spun down granules (142).

Sarcoplasm

Proteins.—The classification of the proteins of the sarcoplasm has been extended mostly to the water-soluble proteins of fishes [(144 to 146); reviews (17, 18, 143)]. Contrary to the uniform behaviour of the contractile proteins the nonenzymatic, especially the electrophoretic, properties of the sarcoplasmic proteins are very different in the muscles of different species. This can be shown when the easily soluble proteins of different kinds of fishes are compared with each other, on the one hand, and with the earlier or more recent findings obtained for mammalian proteins, on the other hand (147). Crystallized beef myoglobin appears to be not quite homogeneous electrophoretically (148).

The conversion of glycogen into hexosediphosphate.—Glycogen degradation seems to concern only the so-called free glycogen. The differentiation between free and bound glycogen is due to a physiological difference and not to an artifact (144).

Phosphorylase-a and -b.—In an extensive and very critical synopsis Cori's starting point is that phosphorylase-*b* needs 5×10^{-6} *M* AMP for a 50 per cent activation. This AMP concentration is not attained in the resting, but only in the working, living muscle. Therefore, Cori suggests that the increase in the decomposition of glycogen during muscular activity is due to the AMP activation of phosphorylase-*b*. An epinephrine concentration of 10^{-7} gm. per mole has the same effect as AMP (150).

Crystallized muscle phosphorylase-*a* is reversibly inhibited by means of *p*-chloromercuribenzoate, which blocks the SH groups, and it is apparently also reversibly split into four fragments (151). Both muscle phosphorylases are not only strongly inhibited by caffeine but also by other methylated oxypurines. This inhibition is removed by an equivalent amount of adenosine or by traces of AMP (152). The attack of the muscle phosphorylase on the glycogen particle is supposed to be an incomplete single-chain mechanism or a multi-chain mechanism, but not a single-chain mechanism.

The enzyme 6-phosphofructokinase can be isolated so as to give a uniform S^0_{20} and D^0_{20} corresponding to a particle weight of 12,000. The preparation is free from other enzymes and it is claimed to contain 3 to 6 per cent of water-

soluble protein and to be an SH-containing enzyme. The enzyme is activated by Mg. Michaelis constants and energy values are given (154, 155). The enzyme is able to form fructose-1,6-diphosphate in the presence of UTP or ITP as well as in that of ATP (156).

Lactic acid formation.—The Meyerhof scheme concerning the formation of glycogen from lactic acid with a simultaneous oxidation of lactic acid is confirmed by means of pyruvate-2-C^{14}: a part of the C^{14} is found as CO_2 and another part as C_3 and C_5 of glycogen (157).

Aldolase prepared from rabbit muscle condenses dihydroxyacetone phosphate on the one hand, and glycolaldehyde and glycolaldehyde phosphate on the other, to the mono- or diphosphate ester of xylulose (159).

From rabbit muscle a new crystallized aldolase has been prepared (158). It splits fructose-1-phosphate (though more slowly), as well as fructose-1,6-diphosphate.

2,3-Diphosphoglyceric acid is formed by means of an appropriate extract of acetone powder prepared from skeletal muscle (160). 2,3-Diphosphoglyceric acid participates in the conversion of 3-phosphoglyceric acid into 2-phosphoglyceric acid, according to Cori.

The proportion of pyruvate to lactate ($=0.06$) remains the same during rest and tetanus in spite of the disparity in the absolute amounts (161). The maximum of $[H^+]$ that limits lactate formation (Meyerhof) is different ac cording to the different muscles (162).

The reactions of energy-rich phosphates.—Berg & Joklik (163) discovered a nucleotide diphosphate kinase (nudiki) which equilibrates the terminal phosphate of a given NTP between the NDP which is present. By this enzyme the recently discovered NTP and NDP are connected with the metabolism and energy change.

Creatine phosphate kinase was crystallized by Kuby, Noda & Lardy (164, 165). From S^0_{20} and P^0_{20} a particle weight of 81,000 was obtained (165). The kinetics and the equilibrium constants of the enzymatic reaction were accurately measured, and from them the thermodynamic values were calculated. The enzyme requires Mg activation. The equilibrium at pH 7 is entirely on the side of ATP, but shifts with increasing pH more and more to the side of CP (166, 167, 168, 170). The creatine phosphate kinase requires free SH groups (168, 169). The creatine phosphate kinase activity of smooth muscles also requires Mg activation and SH groups (170). The equilibrium conditions of the creatine phosphate kinases of smooth and striated muscles are identical (170).

It is shown that creatine phosphate kinase in the presence of ATP phosphorylates creatine and, more slowly, N-ethylglycerocyamine, but not glycocyamine (171).

Such indistinct specifity of creatine phosphate kinase is of significance, because in incompletely extracted muscle carnosine phosphate seems to restore ATP as CP does (172). There are some hints of a phosphorylation and dephosphorylation of anserine as well as of carnosine in the muscular metab-

olism (173, 174, 175). Unlike skeletal muscles of warm-blooded animals, the heart muscles of the latter and lobster muscles do not seem to contain a carnosine-anserine fraction (176).

Myokinase.—Myokinase is now isolated as a uniform preparation with a turnover rate of 210,000 in a noncrystallized (177, 178) and in a crystallized form (179). S^0_{20} is 2×10^{-13} sec. (177), while S^0_{25} is 2.3×10^{-13} (179). S^0_{25} and D^0_{25} indicate a particle weight of 21,000 (179). The more recent data for the pH optimum as a function of the Mg^{++} concentration (180) do not quite correspond to Kalckar's former data. Myokinase is activated by Mg^{++} and contains itself about 20 μM Mg^{++} per gm. tightly bound (180). It is possible that the myokinase activity is not sharply restricted to ADP, for it is reported that an aqueous muscle extract produces reversibly AMP+PPP from ADP+PP. The activity of this reaction in all degrees of purity is strictly proportional, i.e. 1/1000, to the normal myokinase activity (181).

Nucleosidephosphate deaminase.—Similar to the so-called myosin deaminase, sarcoplasm deaminase deaminates ADP as well as ATP (182). In a purified state the deaminase needs no activation by cations. However, its activity is very sensitive to anions and it is inhibited by 50 per cent by 1×10^{-5} *M* Hg^{++} (183). Cysteine reverses the inhibition (183).

Miscellaneous observations.—It is reported that rabbit muscle contains an enzyme that converts CP directly into creatinine+phosphate, provided that glucose-1-phosphate is present (184). Furthermore it is reported that the muscle contains acetylphosphatase activity, which is diminished by a blocking of the NH_2 groups, while the blocking of the SH groups has no effect (185). Arginine phosphate in the crayfish is formed from citrulline and NH_3, provided that ATP is present (186). From fish muscles two riboside hydrolases have been extracted, which split off ribose from nucleosides (187).

Fibril

The single components of contractile proteins.—The contractile proteins of the heart and skeletal muscles of vertebrates are identical or almost so (17, 18). Connell's excellent measurements show that actin, L-myosin, and actomyosin of fishes are identical or almost identical to the corresponding proteins of mammals both in electrical and in chemical properties, and in all the reactions investigated (188, cf. also 17, 18). A few shorter papers covering a smaller sphere confirm this finding for fish muscles (189, 190). A few quantitative deviations of frog myosin are observed and discussed (190a).

The list of invertebrates from whose muscles AM preparations are made has lengthened. These preparations contract on addition of ATP and split it [sea anemone (191), insects (194), *Urechis unicinctus* (197), *Pecten* (198)]. Adenosinetriphosphatase activity is shown for the muscles of *Carcinus maenas* (195). AM solutions of sea anemone (192), *U. unicinctus* (197), and *Pecten* (198) lower the viscosity under ATP. The muscles of *Dendrostomium cymodoceae* exhibit the same x-ray diagram (193) and those of the earth worm the same contractile components of actomyosin, L-myosin, and Y-

protein (196) as mammalian muscles. Thread models prepared from the actomyosin of *Anodonta* cannot be distinguished from those prepared from rabbit psoas in their maxima of tension and shortening and in all other properties (199).

L-myosin particles.—From the flow birefringence of L-myosin solutions variations in particle lengths were calculated in their dependence on pH, ionic strength, and protein concentrations (200, 201).

Relations, which are difficult to explain, between the sedimentation of L-myosin, on the one hand, and temperature and speed of rotation, on the other, cause Parrish & Mommaerts (202) to consider the Svedberg method unsuitable for ascertaining the molecular weight of L-myosin. Laki & Caroll, however, believe they can conclude from similar observations on the diminution of the sedimentation constant with decrease in temperature that the particle weight of 840,000 belongs to a dimer of L-myosin, as the monomer of 420,000 is able to exist only at 0°C. (203). But from their S_0^0 and D_0^0 values a particle weight of 500,000 and not of 420,000 is calculated. However, this explanation does not consider the fact that Portzehl found the mol. wt. of 840,000 also osmometrically at 0°C. with a myosin solution that, during its preparation, was kept at a temperature of about 0°C. (204).

An increase both in phosphate-content and in solubility is ascribed to the addition of PP; a decrease of phosphate-content, however, to the denaturing of L-myosin (205).

The IP and the purification of actins.—According to microcataphoretic studies, the IP of F-actin at low ionic strengths (about 0.07 μ) differs slightly though distinctly from that of the depolymerized actin. The IP for F-actin is at pH 4.8 to 4.9 and for depolymerized actin at pH 5.0 to 5.1 (206). Solutions of G- or F-actin under appropriate ionic conditions can be reversibly precipitated by Mg ions at pH 7 and thus purified (207). The purified actin does not contain any traceable quantities of creatine phosphate kinase, myokinase, pyrophosphatase, deaminase, and hexokinase (208).

The x-ray diagram of paramyosin and actin.—The x-ray diagram has been improved by the R. S. Baer group, as reported in two excellent investigations on the adductors of *Venus mercenaria* (209, 210). An x-ray diagram of isolated F-actin suggests that F-actin belongs neither to Astbury's α- nor β-type (211).

Through purification with resins actomyosin loses almost all its ash, becomes more soluble, and its flow birefringence decreases. The particle weight, however, and the majority of the other properties remain unchanged (212).

The dissociation of actomyosin into L-myosin and F-actin.—The dissociation is inferred from the physical and chemical changes of actomyosin solutions on the addition of polyphosphates. The numerous observations concerning the fact that actomyosin in solution, i.e., under the conditions of the so-called dissociation, splits ATP in precisely the same way as isolated L-myosin does, are extended to investigating the DNP- (213 to 215) and EDTA-effects (216). An activation of the L-myosin adenosinetriphosphatase

by DNP and EDTA is shown to occur at every ionic strength. An activation of the actomyosin adenosinetriphosphatase, however, occurs only at high ionic strength and never at low ionic strength (213, 214, 215), where the actomyosin adenosinetriphosphatase is always inhibited by EDTA (216). The DNP effect on L-myosin at low ionic strength can be diminished step by step by adding actin (213). Dissolved actomyosin adenosinetriphosphatase and dissolved L-myosin adenosinetriphosphatase show identical specificity to the various NTP. Ca- or Mg-activated actomyosin gels, however, exhibit quite a different sequence of specificity (see page 680).

The DNP-activation is not due to the removing of heavy metals or to a binding with ATP (214). It may be added that the UTP splitting is in the same way influenced as the ATP splitting, while the GTP or ITP splitting is inhibited by DNP in any case (215).

Morales' & Blum's assertion that the particle weight of actomyosin solutions determined by light scattering does not decrease on the addition of ATP is not confirmed both for natural and for artificial actomyosin in a careful study by Gergely (217). Perhaps still more unambiguous are the results obtained by A. Weber, who after the addition of ATP to the AM solution has been able to separate actin from L-myosin by ultracentrifugation. She demonstrates by many physical and chemical tests that pure L-myosin remains in the supernatant. On the addition of actin the L-myosin reconverts into actomyosin (218). In the numerous observations on the fine structure of sarcomeres (cf. pages 683–84) actin is shown to be an individual protein, which is found isolated in the I bands of the sarcomeres and bound to L-myosin in the A bands.

The dissociation of actomyosin in solution seems to be unrelated to the contraction induced by NTP, but related to the plasticizing action of all polyphosphates. PP requires higher Mg^{++} concentrations for both the dissociating (219) and the plasticizing effect (220, 221). To the list of polyphosphates that in small concentrations can decrease both the viscosity of AM solutions (222) and the rigidity of AM gels (221) are added CTP, UTP, ITP, and GTP. The viscosity of solutions and the extensibility of fibers seem to be influenced in an analogous way by temperature (223 to 231).

The effect of ATP solutions on light scattering in its dependence on very low ATP concentration has been studied in a new arrangement producing quick results (232). The ATP was continuously restituted by phosphoenol pyruvate plus pyruvate kinase in order to maintain these very low ATP concentrations in spite of the splitting of ATP (233). Thus it is shown that the turbidity decreases even between 10^{-7} up to 10^{-6} *M* ATP and is at a maximum with 10^{-5} *M* ATP (234).

The assertion that the ATP effect on the actomyosin dissociation occurs only when phosphocreatine kinase plus phosphocreatine are present (235) is disproved by Straub's team in stating that the actin, freed from creatine phosphate kinase by Mg. precipitation, reacts as typically as less purified preparations with L-myosin (208). This agrees with the observation that

actomyosin when treated with ATP shows a normal lowering of viscosity even in the absence of guanidino kinase systems (236).

The functional SH and NH_2 groups of contractile proteins.—Under appropriate conditions F-actin can be reversibly depolymerized to G-actin by formaldehyde (237, 238). Thus two NH_2 groups are apparently necessary for polymerizing G-actin to F-actin. The fact that Salyrgan blocks all four SH groups of the G-actin particle, but only two SH groups of the F-actin particle (238), suggests that only two of the four SH groups are required for polymerization (238).

The significance of the SH groups for the adenosinetriphosphatase activity of L-myosin has been examined, using a number of new SH reagents, namely, uranyl chloride (239), Cd^{++}, Ag^{++} (240), and N-ethylmaleimide (241). Both the enzymic activity of L-myosin and the formation of AM on addition of actin disappear in parallel when the SH groups are blocked (240).

The adenosinetriphosphatase activity of L-myosin in the presence of EDTA disappears completely as soon as 50 to 70 per cent of the SH groups are blocked (241), as does the superprecipitation of actomyosin when 50 per cent of the SH groups are blocked (242).

The prosthetic groups of contractile proteins.—The bound ADP of the muscle fibrils does not react with the PC-creatine phosphate kinase system (243, 244). This ADP is a prosthetic group of the actin- and not of the L-myosin component of actomyosin (245 to 247). This has been well proved by investigations of highly purified actin and L-myosin showing that actin contains 18 μmole ADP per gm., while L-myosin is almost free from ADP (246). Actin seems to contain as a further prosthetic group a low-molecular weight substance free from S, N, and P (248).

Ca and Mg must also be considered as prosthetic groups in that they cannot be removed by EDTA. This has been shown partly by an indirect method (249, 250) and partly by a direct analysis of both fibers and muscle brei washed with EDTA (246, 251). Eighty per cent of the firmly bound Ca is bound to actin and only 20 per cent to L-myosin (246). The quantity of Mg bound to actin is about twice as great as that bound to L-myosin (246). Special observations suggest that the Mg bound to L-myosin is at least partially bound to SH groups of L-myosin (241).

The ionic groups of contractile proteins.—Even from a biological point of view these groups must be regarded as functional groups; for by buffering the H^+ liberated both during ATP splitting and during other metabolic processes these groups participate in any case in the energy transfer. They play a decisive role in Morales' theory of contraction; for the neutralization of the opposite charges of AM and ATP is claimed to be a prerequisite for a thermokinetic contraction (24, 252). Consequently, since ATP is negatively charged, a positive charge of AM is supposed.

However, it is still doubtful whether under physiological conditions the excess charge is positive or negative. Recent research workers agree that

when alkaline earths are absent the IP of actomyosin is at pH 5.4 (253) and that of L-myosin at a similar pH (254). While the electrical charge of artificial actomyosin membranes, however, is not changed by Mg (253), the IP of actomyosin solutions, cataphoretically measured, is shifted to pH 8.2 by means of alkaline earths, according to Erdös (254). Both papers apparently agree that myosin is negatively charged in physiological ionic environments (i.e., high concentration of K, low concentration of Mg) (254). The view that ATP and L-myosin, even at pH 5.4, do not have an opposite but rather the same charge is suggested by the observation that the pressure of L-myosin monolayers is increased by ATP without causing a change in the area at minimum compressibility (256, 257). It is claimed that the charge of actin is influenced by alkaline earths and alkalis in the same way as that of L-myosin (254).

It seems as though the optima of precipitation did not always occur at the IP (254, 255). As to whether L-myosin and actin bind K and Na to any extent is a point of discussion (258). On the whole, this field is still much a matter of contradiction although it is the basis of a widely held theory of contraction.

Actin, L-myosin, and tropomyosin, lack N-terminal groups. Tropomyosin, however, has one C-terminal group with an amino acid sequence of Ileu. Ser. Thr. Met. Ileu. Ala. Actin, too, has one C-terminal group: Phe. Ileu. His. 300,000 gm. of L-myosin have one mole of C-terminal groups, namely, Ileu. It is questionable whether Val, Ala, and Leu, are further C-terminal groups (259, 260). Complete amino acid analyses of L-myosin, meromyosins, actin, and tropomyosin, are made by the Bethesda group in very comprehensive studies. Comparisons with known data are on the whole satisfactory (261).

Subunits of L-myosin.—It is confirmed that only H-meromyosin has adenosinetriphosphatase activity and the ability to combine with actin (262). Crystals of L-meromyosin exhibit in electron micrographs sharp bands at distances of 420 Å, which are regarded as the cause of the corresponding distances in the x-ray diagram of the muscle (263). A. G. Szent-Györgyi's discussion concerning the S^0_{20} and D^0_{20} values of the two meromyosins seems to rule out the possibility that the L-myosin particle with a particle weight of 824,000 consists of a double chain of two L-meromyosins each, with the two H-meromyosins at both ends (264). By means of concentrated urea solutions L-myosin is decomposed to particles with the homogenous particle weight of 4600. These particles, however, are not identical. They are highly soluble and are called protomyosins (265). H-meromyosin is not affected by urea (265).

From electrotitration curves, denoting meromyosin in the presence of different salts, a binding of different anions and cations is inferred (266). Only the IP of L-meromyosin is shifted by Mg to the alkaline side (266). This agrees with direct cataphoretic observations (254). The evaluation of the

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electrical groups of unsplit L-myosin from the sum of the electrical groups of the two meromyosins are consistent, but not for all kinds of groups, with the values directly measured by Mihally (267). The digestion by chymotrypsin of L-myosin also yields two kinds of sub-units similar to L- and H-meromyosin, but not identical with them (268).

The Contraction of Actomyosin and NTP Splitting

The conditions of contraction and splitting.—The interactions between actomyosin gel and nucleoside triphosphates have been systematically investigated since the last review (221, 269, 270). Under optimum conditions of activation fiber models in the presence of NTP develop tension corresponding to the following sequence: ATP$\gtrsim$CTP$\gtrsim$Acetyl-ATP$>$UTP$>$ITP $>$GTP$>$ATPP (221, 269). The sequence of the NTP splitting rate is quantitatively the same, with the exception of acetyl-ATP, which is split relatively fast (221). Both the optimum tension developed and the optimum splitting with ATP are three times as high as those with GTP.

The dependence of contraction and of splitting on the NTP concentration is different for the individual NTP. It is remarkable that, unlike ITP and GTP, the NTP with a 6-amino group exhibit substrate inhibition both of tension and of splitting (221, 271). Whether there is an optimum concentration for UTP is a matter of contention (cp. 221 with 271). According to Perry, a substrate inhibition of the ATP splitting occurs always when the ATP concentration is higher than the concentration of Mg present (244, 271). This finding is at variance with former observations made by Hasselbach. It has been shown (272) that Perry's observation is true only of Mg^{++} concentrations $>5\times10^{-4}$ *M*. If the Mg concentration is lower, however, than 5×10^{-4} *M* the ATP concentration can considerably exceed the Mg^{++} concentration before substrate inhibition occurs.

A number of papers written during the period covered by this review confirm that both splitting and contraction are activated by Mg (221, 243, 273). This Mg-requirement increases from ATP to GTP in the same sequence (see above) in which splitting and contraction decrease (221). It is also confirmed that Mg can be replaced by Ca only for splitting, not for contraction (273, 274).

The view that the firmly bound Ca (cf. H III) is also indispensable for contraction is suggested by the observation that a blocking of the bound Ca by EDTA in the presence of excess Mg^{+} leads to relaxation (274, 275). Yet it remains an open question whether this action of Ca in promoting contraction influences the contractile system itself, or the rest of the relaxing factor remaining in the fiber models; for the ITP splitting is not influenced by EDTA blocking Ca, on the one hand (276), and not by the relaxing factor, on the other (221). The pH optimum of model contraction is between 7.5 and 8.0 (277).

The mechanism of ATP splitting.—If ATP is split in the presence of H_2O^{18},

Koshland has shown that O^{18} is to be found almost only in phosphate and not in ADP (278, 279, 280). Thus the breaking up takes place between the terminal phosphate and its bridge oxygen. There is general agreement that ATP splitting in the presence of inorganic P^{32} does not yield ATP^{32} (280, 281). Nor does ATP splitting in the presence of ADP^{32} lead to the formation of ATP^{32} if the splitting is activated with Ca (280, 281). If, however, Mg is used as activator, P^{32} is rapidly equilibrated between ADP and ATP, both in AM sols and in gels (281, 282). The equilibration of P^{32}, even in dissociated AM sols, results from the fact that the exchange of P^{32} is effected only by the actin (281). The rate of P^{32} exchange is not markedly influenced by the presence or absence of L-myosin. L-myosin splits ATP in the presence of ADP^{32} without exchanging P^{32} (281, 282).

The parallelism between contraction and NTP splitting.—The numerous qualitative observations stating such a parallelism have been extended (221, 283 to 286). The quantitative comparison between ATP splitting and power shows that psoas fiber models at 0°C. exhibit the same efficiency as the living muscle (287), according to Hill.

However, the assertion that fibrils and actomyosin gels split ATP substantially more rapidly in the process of shortening than in the subsequent shortened state (288) is based on a wrong interpretation of a true observation. In fact, during the first seconds of splitting, fibrils and actomyosin gels split ATP at three times the normal rate. They do so, however, not only when they are still uncontracted, but also when they have been previously contracted (289). As the living muscle splits the ATP only during the short phase of contraction, this initial burst of ATP splitting can contribute to explaining the higher rate of ATP splitting of the working living muscle (290). Furthermore, the lower tension developed by a muscle fiber when a threshold concentration of ATP was present beforehand (291) may be due to the absence of the initial burst of ATP splitting.

The parallelism between contraction and ATP splitting is not accepted in a few papers (24, 252, 292, 293). If the ionic strength is increased thick bundles of glycerol-water extracted fibers contract more rapidly, though at higher ionic strength the ATP splitting is lower (292). However, the major part of the cross section of such thick fiber bundles is free from ATP and is rigid. Consequently, the bundles shorten about 100 times more slowly than fibril models. The rigid core of such bundles, which is free from ATP, becomes more plastic, however, at higher ionic strengths, and consequently, the speed of shortening becomes higher. It is reported that a model contraction occurs without any splitting of ATP after poisoning with a definite concentration of monoiodoacetate which inhibits contraction incompletely, but splitting completely. It has long been known (22) that pure monoiodoacetate free from I_2 does not inhibit contraction and a reexamination showed that the velocity of splitting is in no way influenced by monoiodoacetate up to a concentration of 5×10^{-3} *M* (294).

The fact known since 1934 (H. H. Weber) that actomyosin models, if denatured by heat, contract like collagen fibers is expanded by the observation that denaturing by Nessler's reagent as well as by SCN^- and I^- is accompanied by a similar irreversible contraction (295, 296). Slight shortening of actomyosin strips (Hayashi), induced by the addition of various hormones, ammonium bases, of alcohols, water, etc., has likewise no connection with physiological contraction, according to the opinion of the authors (297).

The difference between model and vital contraction.—Up to now the most striking difference between these contractions has been the great difference in the speed of shortening between the living cross striated muscle and its fiber model. According to more recent observations, however, these two speeds of shortening seem to be not so very different. While the diaphragm of rats at 37°C. shortens by 1000 up to 1200 per cent of the standard length per sec., (298) fibril models shorten at 20°C. by approximately 200 per cent per sec. (299, 300). These values are to some extent similar, allowing for a reasonable temperature coefficient. Hill's velocity-load law is valid for the psoas fiber model, too (301).

In very careful investigations, Sandow arrives at the result that the monoiodoacetate-induced contracture of the intact muscle is a kind of model contraction and has very little connection with rigor (302). According to Sandow, contracture occurs as soon as the ATP level decreases so far that an accelerated ATP splitting is no longer inhibited by the relaxing factor. In fact, at the maximum of the iodoacetate contracture 40 per cent of ATP remains besides traces of CP, while two hours after the contracture ATP is exhausted (303).

The L-myosin nucleosidetriphosphatase.—The identical activities of actomyosin solutions, on the one hand, and of gels and sols of L-myosin, on the other, are designated as L-myosin nucleosidetriphosphatase activity. The sequence of the Michaelis constants of NTP splitting (304, 305) by L-myosin adenosinetriphosphatase is the same as the sequence of velocities with which the different NTP are split by actomyosin (see page 680). The same is true of the sequence of splitting rates by L-myosin nucleosidetriphosphatase, provided that no alkaline earth is added, regardless of whether the alkaline earths bound to myosin are blocked or not by EDTA (215, 304, 305). If Ca and Mg are present, however, the sequence of the NTP splitting velocities by L-myosin adenosinetriphosphatase is reversed. In this case, GTP and ITP are split more rapidly, but ATP and CTP more slowly, than UTP (215, 304 to 306), although the sequence of the Michaelis constants remains unchanged. This applies both to nucleosidetriphosphatase from dissolved actomyosin (305, 306) and to L-myosin adenosinetriphosphatase (215, 304). The difference in the sequence of the splitting rates in the presence of either free alkaline earths or EDTA is due to the fact that the ATP, CTP and UTP splitting but not the ITP and GTP splitting, are activated by EDTA (215).

A number of kinetic studies confirm and extend our knowledge of the influence of Ca, Mg, K, NH_4, Na, pH, and the substrate concentration

(Michaelis constants), on the kinetics of L-myosin nucleosidetriphosphatase (215, 304 to 308). As a general principle, Mommaerts' point (308) is important that Michaelis constants must be used with some provision for assessing the enzyme substrate affinity, because the decomposition of the myosin-NTP complex occurs often too rapidly.

The acceleration of ATP splitting produced by high pressure (309) confirms in an indirect way the former discovery of Meyerhof & Möhle that ATP splitting is associated with a substantial contraction in volume. The simultaneous increase of the Michaelis constants is considered proof that the formation of the enzyme-substrate binding is associated with an increase in volume. From this rather indirect approach a mutual neutralization of the electrical charges of enzyme and substrate is assumed (309, cf. also 310). The Michaelis constant of the system of myosin and PPP is 150 times as great as that of myosin and ATP. Triphosphate in optimum concentration, on the other hand, is split only one fifth as rapidly as ATP (311). As inorganic PPP produces no contraction, the authors conclude from their finding that contraction is based on the binding of polyphosphates and not on the splitting of polyphosphates. That PPP is not NTP, is not taken into account.

Other enzymatic properties of contractile proteins.—The deaminase activity of actomyosin affects not only AMP but also directly ADP, although with a much lower rate (312, 313). The fact that the same is true of the deaminase of the sarcoplasm (182), and the further fact that the deaminase activity can be separated, partly at least, from myosin (312, 314, 315) suggest that this activity is due to an adsorbed enzyme. This enzyme can be inactivated by SH reagents (315).

The old assertion that myosin in the presence of muscle extract can convert arginine into creatine is replaced by the view that the effective agent of the muscle extract is the ATP (316).

The Localization of the Proteins in the Fibril

X- and Y-protein.—The Y-protein, which is often thought to have some connection with the process of contraction (21), has been recently isolated (317). Furthermore, it has been shown that from pre-extracted fibrils (fibril models), in addition to L-myosin, an X-protein appears to be in the extract. The quantity of this X-protein amounts to 10 to 20 per cent of the total fibril protein (318 to 321). The X-protein is neither actin (318, 319) nor L-myosin (319, 320, 321).

The localization of the proteins.—According to investigations by means of the interference microscope the fibril contains 50 to 55 per cent of its protein as A substance (320). On the other hand, the higher protein density of the A band disappears (10, 323) on the extraction of L-myosin, the quantity of which is also 55 per cent of the fibril protein (321). This suggests that the A substance and L-myosin are identical (cf. also 322, 323). The quantity of X-protein, however, is not sufficient for the A substance. After such extractions

the Z disc, with 6 per cent of the fibril protein, mainly remains in the fibril (320) as well as the actin threads running through I and A (10, 322, 323, 324). Thus the protofibrils of actin and L-myosin definitely overlap, even if it has not been quite decided whether the actin threads lie in the A band between (10, 322) the L-myosin filaments, or inside them (325, 326). In the wing muscle of the common fly the subdivision into A and I bands has been observed by some (325) and has not been observed by others (327) (cf. also 325a). This contradiction may be connected with the fact that the length of the sarcomeres on stretching or contraction changes exclusively at the expense of the I bands (329, 330, cf. also 328). Consequently the I band disappears when the fibril has shortened by 30 per cent (122, 123, 329, 330, 330a). Huxley (122, 123, 329, 330, 330a) has shown this under especially good and unambiguous optical conditions (331).

Relaxing Factors

Thick bundles of fiber models relax from ATP contraction as soon as ATP restitution systems of high concentrations are added. This is observed on the addition of the creatine phosphate kinase system (332 to 335), the phosphocarnosine system (172), the myokinase system (336), and the phosphopyruvate system (337). However, if fibrils (271, 338) or fibers extracted carefully over a long period (339 to 343) are used the ATP restitution systems inhibit neither ATP splitting (271, 338) nor contraction (338). If in this case the ATP concentration is very low, the contraction is even promoted by ATP restitution systems (9, 332). This contradiction shows that the relaxing effect of ATP restitution systems (*a*) is dependent on the special condition in the interior of thick fiber bundles and (*b*) on the inability of the relaxing factor, discovered by Marsh, to inhibit contraction and splitting in the presence of very low ATP concentrations. The reason is that thick fiber bundles always contain the remainder of the relaxing factor and, a much lower concentration of ATP inside than the bath outside. Consequently, the ATP restitution systems increase the ATP concentration inside the fiber bundles to such an extent that Marsh's relaxing factor becomes effective, provided that the bath contains a physiological concentration of ATP. In the case of fibrils, however, the ATP concentration inside and outside is the same from the start, while fibers carefully extracted over a long period lack the relaxing factor (339 to 343). Hence, the factor, which is located in the granules seems to be the only physiological relaxing factor. Its isolation is still in the early stages (340 to 345).

The factor seems to function in the living muscle in the same way as in the models, since microinjection of ATP, UTP, and Mg, are ineffective in living fibers, while Ca injection produces contraction at once by inactivating the relaxing factor (98, 346, 347).

The Importance of NTP for Vital Contraction

ATP splitting is at once reversed in the living muscle by phosphate trans-

fer from CP. Consequently, ATP splitting in the living muscle can be assumed only from the decrease of the sum of ATP+CP. Such a decrease is always found at 20°C. (348 to 355).

Such a decrease, however, is not found at 0°C. when there is a small performance of work (31, 356, 357, 358). Single twitches at 0°C. can be explained by ATP splitting only when a rapid restitution of ATP from an unknown phosphagen is assumed.

The P^{32} content of ATP in muscles that have been in P^{32} solutions for 32 minutes does not change even during intensive muscular work (359, 360). From this the author concludes that the ATP neither undergoes restitution nor even splitting. The same finding has been published by Sacks (361) as early as 1940, using the same arrangement and has been explained by Kalckar and coworkers (362) in 1944 as the result of wrong experimental methods.

Theories of Muscle Contraction

All the theories up to 1954 concerning muscle contraction are discussed critically by Wilkie (25). None of these theories can explain fully the laws that Hill (2, 3 cf. also 4, 5) and his school have found and so well established for the liberation of the extra-energy. More recent modifications of the older theories still do not completely solve this problem (363, 364).

The problem of extra-energy seems to be more easily solvable for those theories that do not suppose a folding of the peptide chains, but a shifting of the actin filaments alongside the L-myosin filaments (26, 329, 365). Moreover, these theories have the advantage in that it can be observed with certainty that the I substance of the one side of the A band disappears, while a substance on the other side of the A band, in the H band, is correspondingly accumulated (see pages 683–84).

The following observation offers an explanation of the shifting: the ATP splitting begins by transferring phosphate to a functional group of F-actin (pages 680–81). Thus this group may be enabled to undergo a sequence of reactions with functional groups of the L-myosin filaments. The functional group of actin may travel in this way from one functional group of L-myosin to the next, shifting thus the actin filament alongside the L-myosin filament (366). This theory removes objections that have been raised to the transfer of energy to AM during ATP splitting (252).

Denervation, Muscle Disease, and Ageing

Denervation.—Some time after denervation, CP, ATP, and organic phosphates decrease (367 to 369). It is reported that the same is true of carnosine, anserine, and creatine, both after denervation (370) and in vitamin E deficiency (371). But also the AM-adenosinetriphosphatase activity (372), as well as both the absolute and the relative amount of AM decrease, while the absolute (but not the relative) amount of connective tissue remains constant (373).

Muscle diseases.—A decrease both in the glycolytic activity (374, 375) and in the glycogen content is observed during myopathy and progressive muscle dystrophy (376). The changes in the phosphate level are not very clear (375, 377); the K content seems to diminish (376). Qualitative alterations of L-myosin are claimed (378).

If during glycogen storage disease glycogen with short outer chains is found amylo-1,6-glucosidase is absent, otherwise it is present (379).

A number of biochemical alterations following a great mechanical or chemical shock are recorded (380 to 383).

Ageing.—Both the ATP and the adenosinetriphosphatase of insect muscles change in a characteristic curve during metamorphosis (384, 385).

In heart and skeletal muscles of mammals the biochemical activity (386, 387, 388) as well as the amount of actomyosin (389, 390) increase before and during birth but decrease during senescence.

Special Properties of Heart, Uterus, and Smooth Muscle

Heart.—The contractile proteins of the heart are quantitatively and qualitatively identical with those of the skeletal muscles or resemble each other closely (391 to 394). There may be small differences in the Mg^{++}, Ca^{++}, and H^{+} optima of contraction (395).

In muscles from hearts with myocardial failure both the amount of actin and the portion of actin in AM apparently decrease (396). It is stated that the CP and the ATP content as well as the rate of oxidative phosphorylation are not significantly different in cardiac muscles from failing and unfailing heart preparations (397 cf. also 398).

Heart glycosides do not influence the viscosity of AM solutions nor the viscosity change under ATP, independently of whether AM is extracted from normal muscles (399) or from cardiac muscles with myocardial failure (396). Glycosides are less bound per gm. of AM than per gm. of serum albumin (400).

Influence of hormones, vitamins, bacterial poisons, and drugs on heart and skeletal muscles.—The muscles of depancreatized rats contain the same amount of ATP, but less of CP, than normal and alloxan diabetic rats (401). It is said that doses of testosterone increase the weight of the heart and its ATP content (402). Vitamin E increases the glycogen content of the diabetic heart but does not influence the glycogen content of the normal heart (403). It is reported that doses of vitamin A, D_2, and K, change the creatine and CP content (404).

Poisoning of living animals with tetanus toxin increases the activity of actomyosin adenosine triphosphatase (405, 406), while doses of toxin *in vitro* are ineffective (406). Diphtheria toxin seems to damage the mitochondria of the heart (407). Quinine, quinidine, and chloroquinine, lower the oxygen uptake as well as the glucose utilization both of heart sections and heart homogenates (408).

Uterus.—The best values for the protein content of virginal uteri seem to be obtained by exhaustive extractions from cow uterus: the protein content being approximately 14 per cent, AM and stroma each 5 per cent, globulin-X fraction 1.5 per cent, and the myogen fraction 2 per cent (409). In infantile uterus the AM content is less, but it becomes greater after treatment with oestrone sulphate (410).

The uterus AM has only 3 to 10 per cent of the adenosinetriphosphatase activity of the AM of the skeletal muscle and a much lower intrinsic viscosity, which, moreover, decreases in a lower degree on the addition of ATP (136). The influence of ionic strength on the adenosinetriphosphatase activity of AM is different for different species. More than 60 per cent of the adenosinetriphosphatase activity of the whole uterus is located in the granules (136).

The qualitative difference between skeletal muscle AM and uterus AM has been the reason for terming the uterus AM an actotropomyosin (411, 412). The tropomyosin component of the contractile uterus protein, however, has been neither crystallized nor exhaustively characterized (411, 412). The glycogen content of the nonpregnant uterus is given as 100 mg. per cent approx. It increases fivefold during pregnancy (413). The O_2 uptake of the uterus is inhibited by epinephrine and growth hormone (414).

Special experiments cause the author to believe that the contractile apparatus of the uterus is directly stimulated by very high voltages and without the help of the membrane (415).

Smooth muscle.—On the whole the biochemistry of smooth muscle agrees with the biochemistry of the cross-striated muscle. However, the phosphagens phosphotaurocyamine and phosphoglycocyamine (65, 66) have at present been found only in a few smooth muscles. Furthermore, it is reported that the squid mantle muscle contains a protein similar to AM, but it cannot be activated by Mg^{++} even at low ionic strength. However, like the mammalian AM, the squid AM splits ATP three times as rapidly as ITP (416).

Miscellaneous.—For the purposes of fish preservation post mortem alterations of fish muscles have been investigated, as well as the composition of red and white muscles of fishes (417, 418, 419). The poisoning of the living muscle by Salyrgan is due to a poisoning of the end-plate, the membrane, and certain metabolic enzymes, but not to a poisoning of actomyosin (420). The myoglobins of different species differ in the type of crystal and according to immunological reactions (421).

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